PREPARATION OF STABILIZED ESSENTIAL FATTY ACID ENRICHED FORMULATIONS FROM MARINE SOURCES FOR USE AS NUTRACEUTICAL AND AQUAFEED SUPPLEMENTS

Thesis submitted in partial fulfillment of the requirement for the degree of

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in

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BY

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Declaration

*I do hereby declare that the thesis entitled "**Preparation of Stabilized Essential Fatty acid Enriched Formulations from Marine Sources for Use as Nutraceutical and Aquafeed Supplements**" is an authentic record of research work carried out by me under the guidance and supervision of Dr. Kajal Chakraborty, Senior Scientist (Organic Chemistry), Marine Biotechnology Division, Central Marine Fisheries Research Institute, Cochin-682018 and the same has not previously formed the basis for the award of any degree or diploma.*

Whenever the work described is based on the findings of other researchers, due acknowledgement is made in keeping with the general practice of reporting scientific observations. However, errors and unintentional oversights, if any are regretted.

Deepu Joseph

Place: Cochin
Date: 24/06/2016

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This is to certify that this thesis entitled ***"PREPARATION OF STABILIZED ESSENTIAL FATTY ACID ENRICHED FORMULATIONS FROM MARINE SOURCES FOR USE AS NUTRACEUTICALS AND AQUAFEED SUPPLEMENTS"*** *submitted by Mr. DEEPU JOSEPH, Junior Research Fellow of Marine Biotechnology Division of Central Marine Fisheries Research Institute, for the award of the degree of Doctor of Philosophy in Chemistry is the result of bonafide research work carried out by him in the Marine Biotechnology Division of Central Marine Fisheries Research Institute in Chemistry, Cochin-682018, under my guidance and direct supervision. I further certify that this thesis or part thereof has not previously formed the basis for the award of any degree, diploma, associateship of any other University or Institution.*

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Chapter 1

INTRODUCTION

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1.1. Polyunsaturated Fatty Acids

The importance of polyunsaturated fatty acids (PUFAs) in human health and nutrition is well recognized. The lipids of the higher organisms contain appreciable quantities of PUFA with methylene-interrupted (homo-allylic) double bonds. Two principal families of PUFAs occur in nature that are derived biosynthetically from linoleic acid (9-*cis*, 12-*cis*-octadecadienoic, LA) and α-linolenic (9-*cis*,12-*cis*,15-*cis*-octadecatrienoic, ALA) acid. Linoleic acid (LA) is the major *n*-6 fatty acid, and ALA is the major *n*-3 fatty acid. In the body, LA is metabolized to arachidonic acid (AA, 20:4*n*-6), and ALA is metabolized to eicosapentaenoic acid (EPA, 20:5 *n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3) (Fig. 1.1). ALA and LA can be sequentially elongated and desaturated through a series of steps involving elongases and delta-5 (Δ^5) - and Δ^6- desaturases (Fig. 1.2). However, though *n*-3 and *n*-6 PUFAs are important components of practically all cell membranes, they are not inter-convertible in the human body. These two PUFA classes compete for the same metabolic enzymes. Since LA is considered the most common dietary PUFA source and is presented in higher amounts than ALA, LA will be the more common substrate for the Δ^6-desaturase. Consequently, it is important to ensure high enough *n*-3 PUFA intake, in order to reduce

LA desaturation and hence the production of AA and eicosanoids derived from this fatty acid. However, the activities of Δ^5- and Δ^6- desaturases are known to be slow in humans and different factors may influence their activities. The saturated fats and cholesterol inhibit their actions, insulin activates the Δ^6- desaturase and the activity of this desaturase is reduced with age. As a whole, human body are unable to synthesize FAs with double bonds in the *n*-3 and *n*-6 positions of the hydrocarbon chain. Therefore, PUFAs should be necessarily introduced into the body through their diet. For this reason, they are called essential polyunsaturated fatty acids (EFAs).

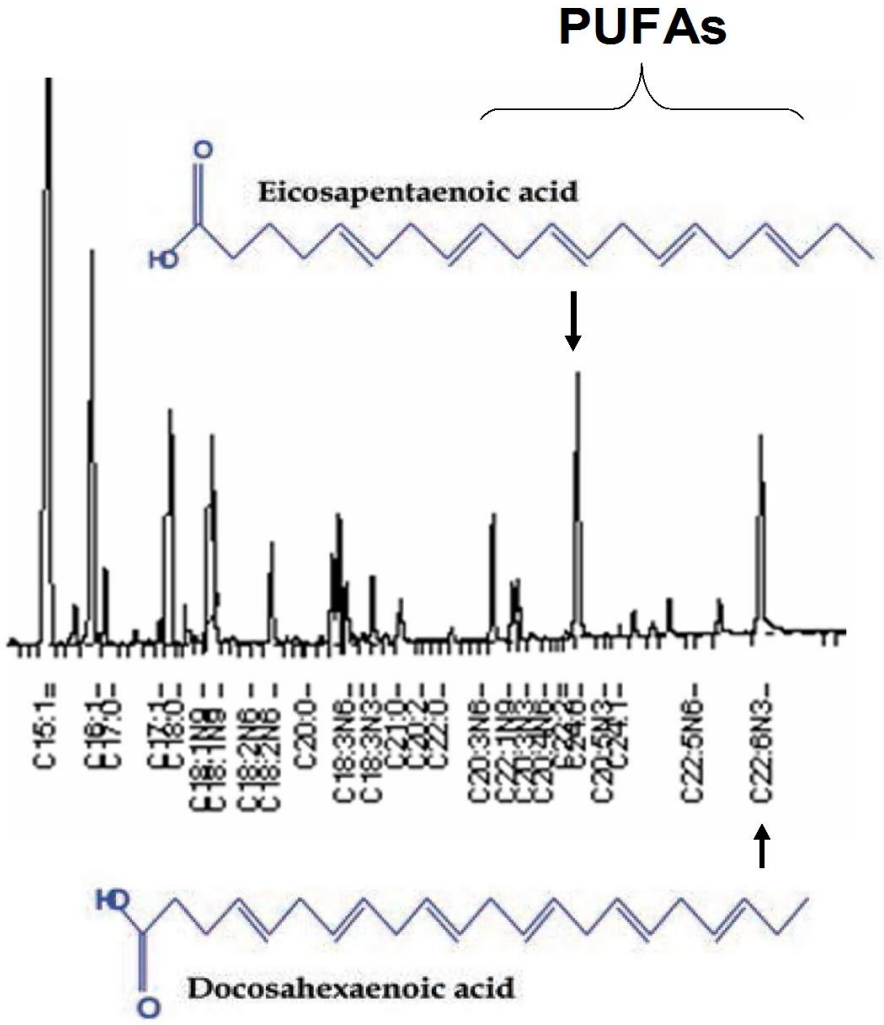
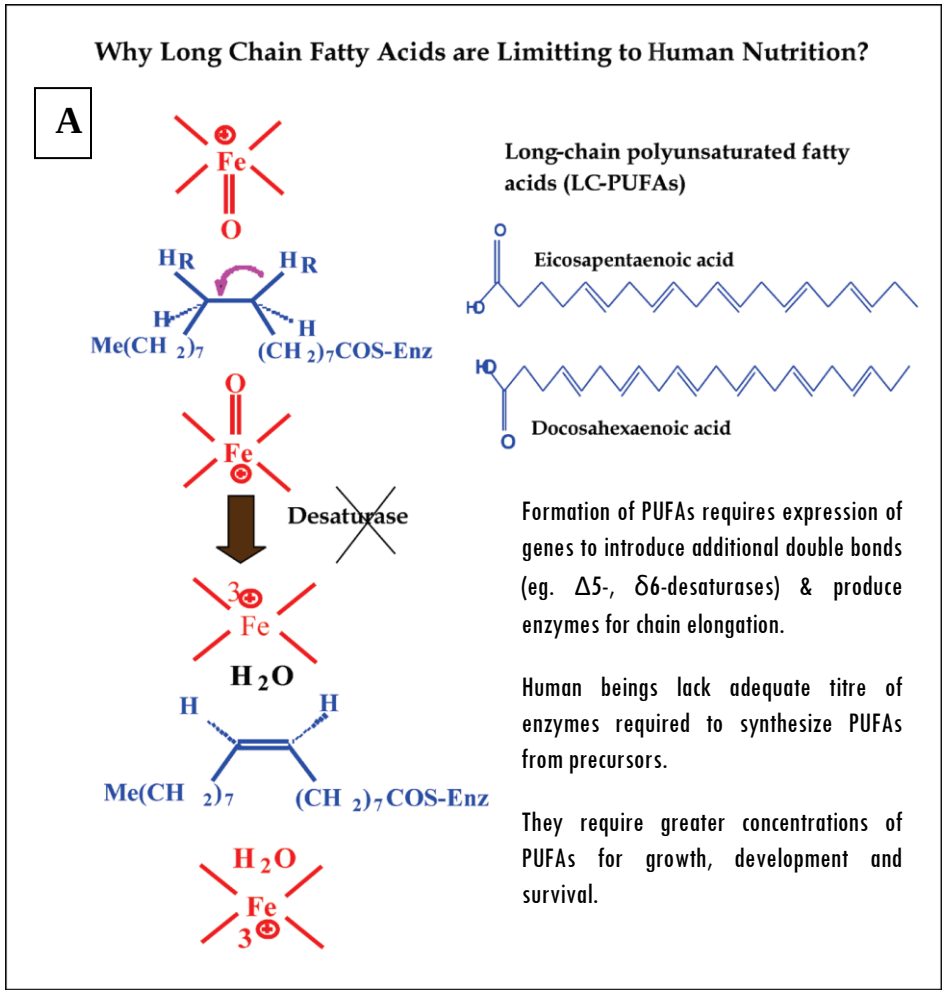


Fig.1.1. An example of chromatogram of fatty acid methyl esters obtained from gas-liquid chromatography. The chemical structures of EPA (20:5*n*-3) and DHA (22:6*n*-3) are also shown.



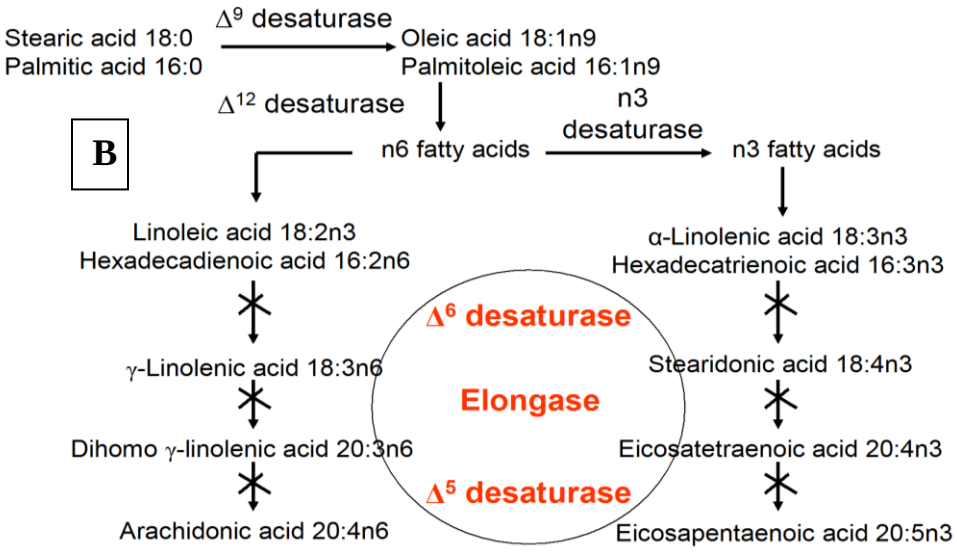


Fig.1.2. The schematic diagrams explaining (**A**) why the long chain fatty acids are limiting to humans, and (**B**) the sequential elongation followed by desaturation of LA (18:2*n*-3) and ALA (18:3*n*-3) through a series of steps involving elongases and delta-5 (Δ^5)- and Δ^6desaturases

1.2. Health Benefits of Essential Polyunsaturated Fatty Acids

In the past two decades there has been great interest in the nutritional value of fish as the evidence of the unique health benefits of *n*-3 long chain polyunsaturated fatty acids (LC-PUFA) is continually increasing. An increased interest in the potential health effects specifically of *n*-3 fatty acids started in the 1970s. This was initiated mainly by epidemiological findings of Bang *et al*. (1971) who discovered that Greenland eskimos have an extremely low incidence of cardiovascular disease (CVD), despite their traditional diet rich in fat, saturated fatty acids and cholesterol (Fig.1.3). The phenomenon was explained by the high dietary intake of very long chain *n*-3 PUFA from fish and other marine animals.

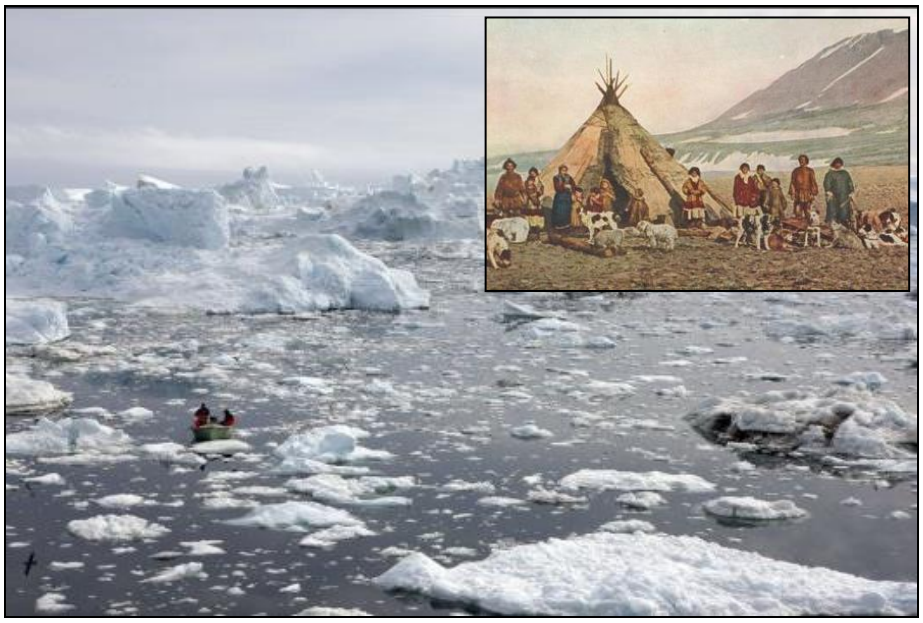


Fig.1.3. Eskimos dangles a fishing line into the water (Inset: An Eskimo family)

This finding stimulated global research on *n*-3 PUFA and resulted in a large number of epidemiological, animal and clinical studies investigating the potential beneficial role of *n*-3 PUFAs, especially EPA and DHA (Valsta *et al*., 1996). Discernable health implications from the consumption of these *n*-3 fatty acids (EPA & DHA) have been reported (Simopoulos 1994; Haumann 1997). At sufficient levels of incorporation, EPA and DHA influence the physical nature of cell membranes and membrane protein-mediated responses, eicosanoid generation, cell signaling and gene expression in many different cell types. Through these mechanisms, EPA and DHA

influence cell and tissue physiology, and the way cells and tissues respond to external signals. In most cases, the effects seen are compatible with the improvements in disease biomarker profiles or in health-related outcomes. As a result, very long chain *n*-3 fatty acids play a major role in achieving optimal health and in protection against disease. EPA is the precursor of prostaglandins, thromboxanes and leukotrienes. DHA is a component of the phospholipid membrane of brain and retina cells; consequently, it is essential for human health (Fournier *et al*., 2007). These *n*-3 PUFAs are known to present anti-inflammatory and immunomodulating effects as well as chronic diseases involving the inflammatory processes (Wall *et al*., 2010; Simopoulos, 2002; Mori & Beilin, 2004). Recent studies have identified novel families of mediators formed from EPA and DHA, termed E- and D-series resolvins, respectively. These mediators are derived from *n*-3 LC-PUFAs *viz*., EPA and DHA produced via the cyclooxygenase and lipoxygenase reactions, and are considered to be anti-inflammatory (Calder 2006; Serhan *et al*., 2002). Beneficial health effects of these *n*-3 PUFAs are well demonstrated, mainly in the prevention of cardiovascular diseases.

1.3. Essential Polyunsaturated Fatty Acids and Human Diet

The diet of our ancestors was less dense in calories, being higher in fiber, rich in fruits, vegetables, lean meat, and fish. As a result, the diet was lower in total fat and saturated fat, which contained as much as ~5-6 g/day of *n*-3 PUFAs with a high EPA and DHA proportion. From an evolutionary point of view, there has also been a rapid increase in consumption of foods rich in *n*-6 PUFAs and a decrease in the intake of *n*-3 PUFAs in Western societies during the past 150 years. These resulted in an increased *n*-6 to *n*-3 ratio (*n*-6/*n*-3), ranging from 10 - 20:1 as compared to 1:1 in the ancestral diet. These two classes of PUFA should be distinguished because they are metabolically and functionally distinct and have opposing physiological functions; their balance is important for homeostasis and normal development. However, an excess of *n*-6 fatty acids can create an imbalance of the ratio and result in pro-inflammatory response, propagation of cancer, heart disease, stroke, etc (Connor 2000; Calder 2008). Hence, a balanced *n*-6/*n*-3 ratio in the diet is essential for normal growth and development and should lead to decreases in cardiovascular

disease and other chronic diseases and improve mental health. Different recommendations exist regarding EPA and DHA intake. The United Kingdom Department of Health recommends intakes of 1.5 g of EPA plus DHA per week (i.e. approximately 0.2 g per day), i.e. equivalent to two servings of fatty fish, for healthy subjects (UK Department of Health 1994). The Danish Veterinary and Food Administration recommend eating fatty fish at least twice a week (FVST 2006), whereas other countries such as Canada, Sweden, the United Kingdom and Japan recommend a daily intake of EPA and DHA at a level around 0.3 - 0.5 g (Kris-Etherton *et al.*, 2002). Similarly, European Food Safety Authority (EFSA) recommended a daily reference intake value for EPA and DHA at 0.25 g (Heller 2009). For general cardioprotection, the American Heart Association (AHA) recommends about 1 g of EPA/DHA daily for patients with known coronary heart disease (Kris-Etherton *et al.*, 2002). For people with no known heart disease, the AHA recommends eating oily fish at least twice a week, or about 500 mg of EPA/DHA per day. Recommendations of the International Society for the Study of Fatty Acids and Lipids (ISSFAL) suggest an adequate intake of n-3 PUFA to be 0.65 g of DHA plus EPA per day (minimum 0.22 g per day each) and 1.0 g of ALA per day (Simopoulos 1999).

1.4. Marine Fish as a Source of Essential Polyunsaturated Fatty Acids

An absolute requirement is necessary for the EFAs for growth, reproduction and good health. EPA and DHA are synthesized mainly by both uni and multicellular marine plants such as phytoplankton and macro algae. They are eventually transferred through the food web and are incorporated into lipids of aquatic species such as fish and marine mammals, particularly those living at low temperatures, because their physiology, environment and diet promote *n*-3 fatty acid production in their flesh. Hence, an increase in *n*-3 PUFA intake could be achieved by promoting fish consumption and increasing the use of ALA rich oils instead of vegetable oils which contain mainly *n*-6 fatty acids (mainly LA, 18:2*n*-6) and animal fat containing *n*-9 fatty acids (mainly oleic acid, 18:1*n*-9). Fish oils are considered the major commercial

source of EPA and DHA, and have received much attention in the research and commercial aspects because of their positive role in human health (Fournier et al. 2007). Therefore increased consumption of marine lipids has been suggested in order to increase the intake of *n*-3 fatty acids in our diet.

1.5. Why Purified form of *n*-3 Fatty Acids is Required?

A substantial increase in daily intake of *n*-3 PUFA rich foods is needed in order to meet even the lowest recommendations. However, in order to use the PUFAs as ingredients for nutraceutical formulations, they are in need of isolation in a high purity. Saturated fats are not necessary for body but also bring health disadvantages in that they get fat and increase cholesterol and triglyceridemia. Saturated fatty acids (palmitic, stearic), is digested and absorbed hard and can create various digestive problems. Also, the triacylglycerol (TAG) structure in the crude lipids from fishes limits the content of *n*-3 PUFA (mostly EPA and DHA) to maximum 300 g/kg. Higher concentrations are only achieved by the separation of fatty acids from the triacylglycerol structure. The low-temperature crystallization, enzymatic splitting, fractional and molecular distillation, chromatographic methods, supercritical fluid extraction and urea complexation methods are currently practiced for preparation of PUFA concentrates from marine oils. Selective enzymatic hydrolysis of saturated fatty acids which would concentrate the *n*-3 PUFA in the acylglycerol form is a recent biotechnological advance that may be extended to large scale operations once economical constraints are addressed. The *n*-3 PUFA concentrates can also be in the form of free fatty acids, methyl and ethyl esters (Shahidi & Wanasundra 1998). However, despite of their health benefits, highly unsaturated fatty acids are very sensitive to oxidative deterioration and thus create practical problems.

1.6. Stability of Fish Oils

Lipid oxidation is influenced by many factors: the medium, oxygen concentration, temperature, light, degree of unsaturation, and metal ions among others. Lipids containing high levels of PUFA, due to their high unsaturation in the acyl chain, which are highly susceptible to oxidation (Kamal-Eldin & Yanishlieva

2002). Oxidation of lipids not only produces rancid odors and flavors, but also can decrease nutritional quality and safety by the formation of primary and secondary oxidation products (hydroperoxides, free radicals, epoxides, etc). A large number of saturated and unsaturated aldehydes, ketones, acids, and other products have been isolated from oxidized oils, and have been shown to contribute to the undesirable flavors and odors. These products of lipid oxidation are known to be health hazards since they are associated with aging, membrane damage, heart disease and cancer (Suja *et al*., 2004). The production of biologically active carbonyl compounds including acrolein, malonaldehyde (MDA) and 4-hydroxyl-2-nonenal (4-HN) from lipids during oxidation (Miyake & Shibamoto 1996). The initiation of free radical formation involved in oxidative processes of the oil requires low activation energies (4.14 kcal/mol) and their rates are not changed significantly by lowering of the storage temperature (Labuza 1971). Therefore, it is essential not only to prepare a PUFA concentrate, but to optimize their stabilization in an attempt to increase their shelf-life, and to retain their original properties.

1.7. Antioxidants to Stabilize Fish Oil

The most widely used antioxidants are free radical scavengers that remove reactive radicals formed in the initiation and propagation steps of autoxidation. Fish oils are usually stabilized with both synthetic and natural antioxidants. These antioxidants are mainly phenolic compounds, which are able to compete, even at low concentrations, with lipid molecules as hydrogen donors to hydroperoxy and alkoxy radicals, producing hydroperoxides and alcohols and an unreactive radical which do not initiate or propagate the chain reaction. However, today, there is a decline in the use of synthetic antioxidants such as, butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA), *tert*-butyl hydroquinone (tBHQ) and propyl gallate, due to the questions regarding their food safety and toxicity. Thus, natural ingredients are progressively replacing synthetic antioxidants and new, efficient natural antioxidant blends are being developed. The choice of natural antioxidants to stabilize marine oils for human consumption is earlier restricted to a few substances with α-tocopherol (or its synthetic analog) being most frequently used. Although

tocopherols are considered as safe natural antioxidants they do not always provide effective protection against *in vitro* oxidation of fish lipids. Therefore recently, research on other natural antioxidants has gained momentum as they are considered to pose no health risk to consumers and several natural sources are being studied. Numerous studies have focused on natural antioxidants in terrestrial plants such as rosemary, sage, green tea, oregano, clove, basil to prevent lipid oxidation (Suja *et al.*, 2004; Wanasundara & Shahidi 1998; Lee & Shibamoto 2001; Balasundram *et al.*, 2005). Antioxidative compounds derived from marine sources may work synergistically with fish oils to produce a stable product, in particular, functional compounds, such as tocopherols (Airanthi *et al.*, 2011).

1.8. Enrichment of the Bioactivity of Purified Fish Oil

As a whole, the intake of *n*- 3 fatty acids has been linked to promotion of human health to fight against numerous diseases. However, the complex methods for the purification of these *n*-3 fatty acids might make them to reduce its bioactive properties. These properties can be modified by adding other marine derived components, which possess potential bioactive properties. The marine environment comprises complex ecosystems and many of the organisms are known to possess bioactive components as a common means of self-defense or for the protection of eggs and embryos. Recently, many bioactive compounds have been extracted, characterized and purified from various marine animals such as bacteria, fungi, algae, dinoflagellates, tunicates, sponges, soft corals, bryozoans, cephalopods and which possess anti-inflammatory, antibiotic, anti-parasitic, antiviral and anti-cancer activities echinoderms (Donia & Hamann 2003; Haefner 2003).

1.9. Objectives

The scope of the present work lies to prepare a comparatively cheaper import-substitute PUFA concentrate from locally available low-value fish and fishery by-catch. This work also aims to develop a process to prepare a stabilized formulation containing enriched PUFAs and individual or combination of different ingredients

with potential antioxidant properties for use in food/feed applications as nutraceuticals, including dietary supplements, and animal/aquafeed products.

Based on this background the objectives of the thesis are as follows:

- To screen various marine fishes for higher content of polyunsaturated fatty acids (PUFAs).
- To prepare the fatty acid concentrate(s) by chemical hydrolysis of triglycerides from the selected marine source with high *n*-3 PUFAs followed by various physico-chemical/biotechnological procedures like amide complexation, enzymatic hydrolysis and/or chromatography based on differences in physico-chemical properties associated with the number of olefinic double bonds or acyl chain length.
- To study the shelf life of the refined oil/concentrated fatty acids and purified fatty acid methyl esters with regard to lipid peroxidation status, and reactive oxygen species (ROS) scavenging activity.
- To purify and structurally characterize components with antioxidant activities from different natural sources, especially marine sources and to shortlist potential antioxidant molecules/components.
- To prepare stabilized PUFA concentrate(s) by the addition of individual or combination of different selected antioxidant ingredients with potential antioxidant properties in an attempt to impart stability for health food/aquafeed supplements.
- To enrich the bioactivity with respect to anti-inflammatory property of the purified fish methyl esters using marine bioactive ingredients.
- To prepare a formulation containing purified PUFA esters and suitable additives for use in food applications as nutraceuticals, including dietary supplements, and animal/aquafeed products.

1.10. Thesis Outline

Based on the above objectives the present thesis is crystallized into a total of eleven chapters. The background and importance of the study with objectives are discussed and explained in the Introduction under Chapter 1. Chapter 2 deals with the detailed review of the works carried out regarding the significance of fish oil, chemistry and composition of marine oils, applications and uses of fish oils, processing of fish oils, degradation of fish oils, analysis of lipid oxidations, how to control their oxidative degradation, selection of antioxidants and enrichment of fish oils. Subsequently, the screening of marine fishes based on their seasonal variation in fatty acid composition and selection of the best species is described in Chapter 3. Chapter 4 describes the extraction and refining of triacylglycerides from Indian Oil Sardine S*ardinella longiceps*. The processing of the refined fatty acid to their purified methyl ester form is detailed in Chapter 5. In order to stabilize the purified fatty acid methyl esters, selected marine additives were studied for their antioxidant activities. The bioactivity profile and the preparation of these additives are detailed in Chapter 6. The effects of antioxidant additives on the shelf-life of the refined oil, concentrated and purified methyl esters are described in Chapter 7, which also compared the antioxidant activities of the marine additives and natural herbs to prevent the oxidative degradation of the purified fish oil. In Chapter 8, the isolation and characterization of bioactive secondary metabolites from potential marine additives, which found to be the most effective additives for imparting oxidative stability to the sardine oil/esters, were evaluated. To further enhance the bioactivity of the purified methyl esters, different marine cephalopod extracts were prepared from different sources and a potent species based on their anti-inflammatory profile was short-listed and detailed in Chapter 9. An enriched PUFA formulation for use as nutraceutical and aquafeed supplements was prepared and their studies were described in Chapter 10. Chapter 11 summarized the entire work carried out in the present study.

Chapter 2

REVIEW OF LITERATURE

Contents

2.1. Chemistry and Composition of Fish Oils

Fish oil is the lipid fraction extracted from fish/fish by-products, which contain saturated, monounsaturated and polyunsaturated fatty acids. Saturated fatty acids (SFAs) do not possess olefinic bonds in hydrocarbon chain. Examples of SFAs are lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid, behenic acid, and lignoceric acid. Monounsaturated fatty acids (MUFAs) possess one double bond, the typical examples being myristoleic acid, palmitoleic acid, elaidic acid, oleic acid, erucic acid, and nervonic acid. Fatty acids with two or more than two double bonds are termed as PUFAs. The PUFAs of four to six double bonds are a characteristic for marine fish oil, resulting in the unique health property often referred as the *n*- 3 and *n*-6 fatty acids. The first element of long chain *n*-3 fatty acids is ALA (C18:*n*-3); other members of this family are derivatives of ALA with longer, more unsaturated hydrocarbon chains such as EPA, DHA etc. EPA, systematically called all-*cis*-5, 8, 11, 14, 17-eicosapentaenoic acid is a carboxylic acid with a 20-carbon chain with five *cis* double bonds (denoted as C20:5*n*-3). DHA, systematically called as all-*cis*-docosa-4,7,10,13,16,19-hexaenoic acid is a carboxylic acid with a 22-carbon chain and six *cis* double bonds (denoted as C22:6*n*-3) (Fig. 2.1). The *n*-3 fatty acids are said to be beneficial in conditions from Alzheimer disease to Zellweger syndrome. There are solid and compelling evidence that EPA and DHA play vital physiological role in cardiovascular health, visual function and in the inflammatory process.

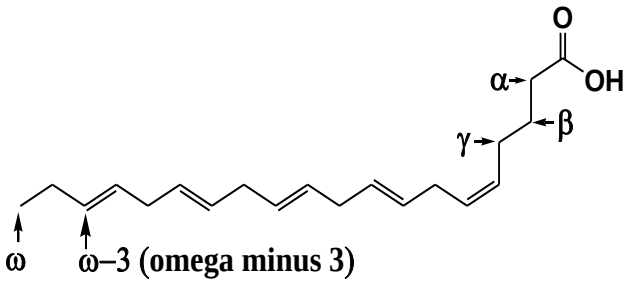


Fig. 2.1. Chemical structure of EPA (*cis*-5,8,11,14,17-eicosapentaenoic acid, 20:5*n*-3. The first carbon following the –COOH group is designated as α-carbon; the next one is β-carbon, and the most distant carbon to carboxyl group is designated as *n* (ω)th carbon. The carbon atoms close to *n*-carbon are designated in relation to it. For example, the third from the end is *n*-3 (omega minus 3), and sixth from the end is *n*-6 (omega minus 6).

EPA and DHA are found almost exclusively in marine oils. Fish do not produce EPA and DHA. Rather, these oils are synthesized by single-celled marine organisms that fish eat. Marine life higher in the food chain accumulates more *n*-3 oils than fish lower in the food chain (Fig. 2.2). Therefore fish such as salmon, sardines, mackerel etc are richer in *n*- 3 fatty acids than fish lower in the food chain. Freshwater fish that have not been in the ocean contain very little *n*-3 fatty acids because the algae that contains *n*-3's does not exist in freshwater. Therefore, some popular fish species such as farm raised tilapia and catfish contain very low amounts of *n*- 3 fatty acids (Science Daily, 2008).

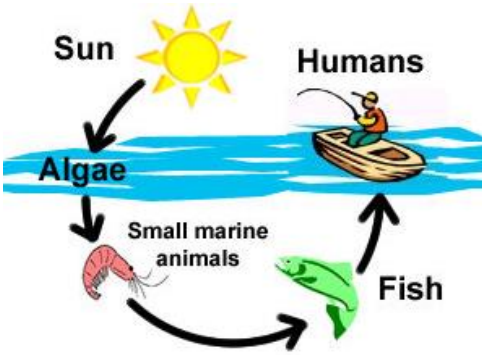
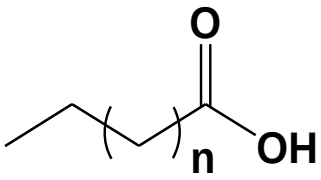
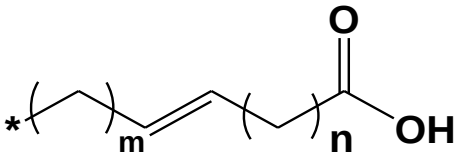
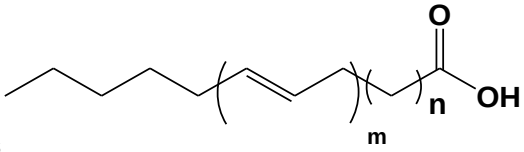
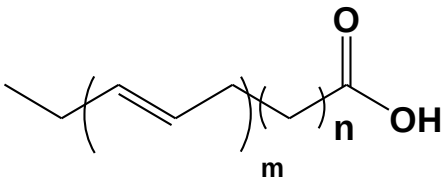


Fig. 2.2. Schematic representation of marine food chain

The common names of the fatty acids (SFA, MUFA and PUFAs) with abbreviated formulae, molecular formulae, and molecular weight are detailed under the Table 2.1.

Table 2.1 Fatty acids and their abbreviated formulae, molecular formulae, and molecular weight

			Abbreviated nomenclature		Molecular formulae	Molecular weight
Saturated fatty acids						
	m	n	**With respect to-COOH group**	**With respect to n (or ω)-group**		
Myristic acid	0	11	14:0	14.0	$C_{14}H_{28}O_2$	228.37
Pentadecanoic acid	0	12	15:0	15.0	$C_{15}H_{30}O_2$	242.4
Palmitic acid	0	13	16:0	16.0	$C_{16}H_{32}O_2$	256.42
Heptadecanoic acid	0	14	17:0	17.0	$C_{17}H_{34}O_2$	270.45
Stearic acid	0	15	18:0	18.0	$C_{18}H_{36}O_2$	284.48
Arachidic acid	0	17	20:0	20.0	$C_{20}H_{40}O_2$	312.53
Heneicosanoic acid	0	18	21:0	21.0	$C_{21}H_{42}O_2$	326.53
Behenic acid	0	19	22:0	22.0	$C_{22}H_{44}O_2$	340.58
Tricosanoic acid	0	20	23:0	23.0	$C_{23}H_{46}O_2$	354.61
Lignoceric acid	0	21	24:0	24.0	$C_{24}H_{48}O_2$	368.64
Monounsaturated fatty acids						
						
Myristoleic acid	2	7	14:1 Δ^9	14:1*n*9	$C_{14}H_{26}O_2$	226.36
Cis-10-Pentadecenoic acid	2	8	15:1 Δ^{10}	15:1*n*10	$C_{15}H_{28}O_2$	240.36
Palmitoleic acid	4	7	16:1 Δ^9	16:1n9	$C_{16}H_{30}O_2$	254.41
Cis-10-Heptadecenoic acid	5	8	17:1 Δ^{10}	17:1*n*10	$C_{17}H_{32}O_2$	268.43
Elaidic acid	7	7	18:1 Δ^9	18:1*n*9 trans	$C_{18}H_{34}O_2$	282.46
Oleic acid	7	7	18:1 Δ^9	18:1*n*9 cis	$C_{18}H_{34}O_2$	282.46
Cis-11-eicosenoic acid	7	9	20:1 Δ^{11}	20:1*n*11	$C_{20}H_{38}O_2$	310.51
Erucic acid	7	11	14:1 Δ^{13}	22:1*n*13	$C_{22}H_{42}O_2$	338.57
Nervonic acid	7	13	14:1 Δ^{13}	24:1*n*15	$C_{24}H_{46}O_2$	366.62
***n*-6 Polyunsaturated fatty acids**						
						
Linolelaidic acid	2	6	18:2 $\Delta^{9,12}$	18:2*n*6 *trans*	$C_{18}H_{32}O_2$	280.45
Linoleic acid	2	6	18:2 $\Delta^{9,12}$	18:2*n*6 *cis*	$C_{18}H_{32}O_2$	280.45

γ-Linolenic acid	3	3	18:2 $\Delta^{6,9,12}$	18:3*n*6	$C_{18}H_{30}O_2$	278.43
Cis- 11,14-Eicosadienoic acid	2	8	20:2 $\Delta^{11,14}$	20:2*n*6	$C_{20}H_{36}O_2$	308.5
Cis- 8,11,14-Eicosadienoic acid	3	5	20:2 $\Delta^{8,11,14}$	20:3*n*6	$C_{20}H_{34}O_2$	306.48
Arachidonic acid	4	2	20:2 $\Delta^{5,8,11,14}$	20:4*n*6	$C_{20}H_{32}O_2$	304.47
Cis-13,16-Docosadienoic acid	2	10	22:2 $\Delta^{13,16}$	22:2*n*6	$C_{22}H_{40}O_2$	336.55
***n*-3 Polyunsaturated fatty acids**						
						
Linolenic acid	3	6	18:2 $\Delta^{9,12,15}$	18:3*n*3	$C_{18}H_{30}O_2$	278.43
Cis-11, 14, 17- Eicosatrienoic acid	3	8	20:2 $\Delta^{11,14,17}$	20:3*n*-3	$C_{20}H_{34}O_2$	306.48
Cis-5, 8, 11, 14, 17- Eicosapentaenoic acid	5	2	20:2 $\Delta^{5,8,11,14,17}$	20:5*n*-3	$C_{20}H_{30}O_2$	302.45
Cis-4, 7, 10, 13, 16, 19- Docosahexaenoic acid	6	1	22:2 $\Delta^{4,7,10,13,16,19}$	22:6*n*-3	$C_{22}H_{32}O_2$	328.49

'm' represents the number of double bonds and 'n' represents the number of carbons

The crude fish lipids generally occurring as esters of the trihydroxyl alcohol, i.e. TAGs with 1, 2 or 3 long chain carboxylic acids (mono, di and tri acyl glycerols, respectively) or free fatty acids (Fig. 2.3), also constitutes glycerol ethers, mixed triglycerides, fatty alcohols (such as wax esters), sterol (as sterol esters), phospholipids. Most natural TAGs do not have a random distribution of fatty acids on the glycerol backbone. In plant oils, unsaturated acids predominate at the sn-2 position, with more saturated acids at sn-1 and sn-3. The distribution of fatty acids at the sn-1 and sn-3 positions is often similar, although not identical. In animal fats, the type of fatty acid predominating at the sn-2 position is more variable; for example, palmitate may be selectively incorporated as well as unsaturated acids.

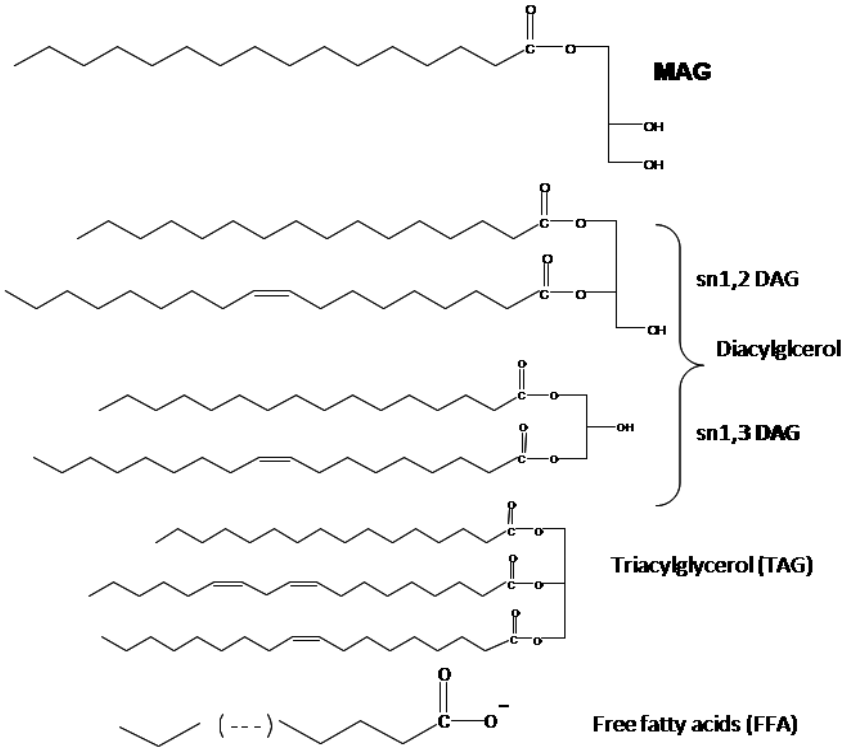


Fig. 2.3. Structures of monoacyl glycerol MAG, diacyl glycerol DAG, triacyl glycerol and free fatty acids

2.2. Applications of Fish Oil

The PUFAs in the fish oil have multiple roles in the human body as detailed in the following sections.

2.2.1. Fatty Acids and their Importance in Human Health and Nutrition

Large amounts of PUFA are required for cellular membrane structure and function, as they are integral elements of phospholipids that are the fundamental components of lipid bilayers. LC-PUFAs have roles in regulating cellular metabolism and more importantly, dioxygenase-catalysed oxidation of LC-PUFA to yield bioactive eicosanoids. Every tissue in human body produces bioactive eicosanoids having a wide range of physiological actions, immune and inflammatory responses, cardiovascular, renal and neural functions, and reproduction process. The important natural sources of *n*-3 LC-PUFAs are marine fish *viz.*, sardine, mackerel, tuna, ribbonfish, grouper, and shark, which contain PUFA levels of about 30%. For this reason, marine fish oils are preferentially used as food items for better health (Vazhappilly & Chen 1998;

Mansour *et al.*, 2005). Fig 2.4 shows the schematic diagram showing the importance of marine fishes in human diet.

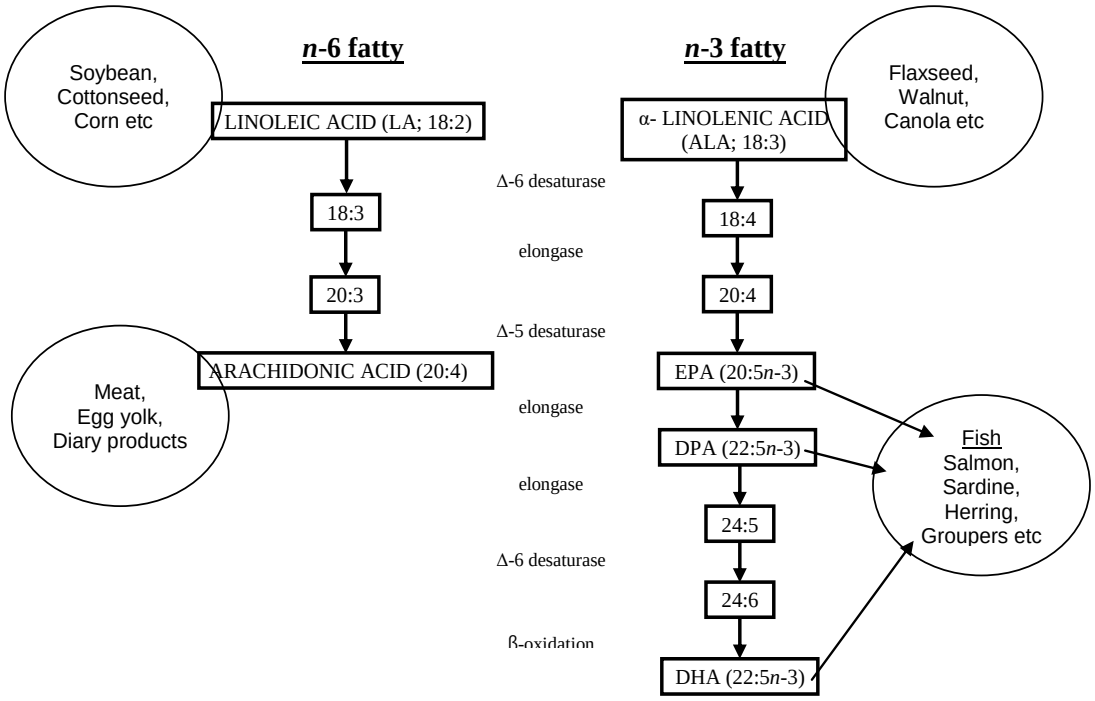


Fig. 2.4. Schematic diagram showing the importance of marine fishes in human diet. DPA – Docosapentaenoic acid; EPA- Eicosapentaenoic acid; DHA – Docosahexaenoic acid

DHA maintains structural and functional integrity in cell membranes in addition to the neural development and function, while AA and EPA are involved in, respectively, the production and modulation of eicosanoids (Brown 1994). AA has been an essential function of producing eicosanoids, making it an essential fatty acid, which has to be provided in diets because prostaglandins (PGF_2R) are produced from 20:4*n*6, and has roles in several biochemical and physiological functions. AA is the basis for cyclooxygenase (COX) action to produce PGF_2. AA, being a major component of phosphoinositol, was reported to have a vital role in the transduction signal mechanism. Many of those from AA are pro-inflammatory, which serves a purpose in immune reactions to bacteria, oxidation, or injury to tissue. It has been established that EPA modulates eicosanoid production from AA, and failure to supply these two fatty acids in the appropriate balance may result in adverse biochemical responses. Research on exploring sources LC-PUFAs, *viz.*, DHA, EPA, and AA for use in nutrition have received considerable attention. The importance of

PUFAs in human nutrition has been extensively investigated during the past 20 years. DHA, which is a vital component of the phospholipids of cellular membranes, especially in the brain and retina, is necessary for their proper functioning. The *n*-3 fatty acids favorably affect atherosclerosis, coronary heart disease, inflammatory disease, and perhaps even behavioral disorders. Membrane fluidity is essential for proper functioning of these tissues. The *n*-3 fatty acids are essential fatty acids, necessary from conception through pregnancy and infancy and, undoubtedly, throughout life. An imbalance in *n*-3/*n*-6 ratio can accentuate *n*-3 fatty acid deficiency state.

2.2.2. Fatty Acids and their Role to Prevent Cancer

Among dietary factors postulated to influence cancer development are long chain polyunsaturated *n*-3 fatty acids, found in fish. Earlier studies revealed inverse relation between marine fatty acid consumption and mortality rates of prostate (Hebert *et al.*, 1998) and breast cancer (Hebert *et al*., 1996). The mechanisms proposed how the intake of marine fatty acids might lower the risk of cancer are the inhibition of eicosanoid biosynthesis from AA, *n*-6 fatty acid. Prostaglandins converted from AA by the COX-2 enzyme, notably PGE_2, have been linked to carcinogenesis *viz.*, mammary tumor development, proliferation of breast and prostate cancer (Erickson, 1986). Tumor cells typically produce large amounts of AA-derived PGE_2, which may impede immune system function, possibly through their role in the generation of suppressor T cells (Erickson, 1986). Marine fatty acids were reported to inhibit COX-2 and the oxidative metabolism of AA to PGE_2. EPA and DHA also inhibit lipoxygenases (LOXs) which metabolize AA to HETEs and leukotrienes. 12-HETE has been linked to the suppression of apoptosis, stimulation of angiogenesis, stimulation of tumor cell adhesion, and expression of the invasive phenotype. It is apparent that both EPA and DHA can inhibit the biological activity of eicosanoids and androgens (Liang & Liao 1992), which are known to have a stimulating effect on cell growth and uncontrolled cell proliferation (Ghosh & Myers 1997). It is well established that in animal models and in human cancer cell lines, EPA and DHA were found to suppress cell growth.

2.2.3. Role of Fatty Acids to Combat Atherosclerosis and Cardiovascular Diseases

Researchers in the USA and Sweden followed 39,367 Swedish men, aged between 45-79, from 1998 to 2004. They recorded details of the men's diet and tracked the men's outcome through Swedish inpatient and cause-of-death registers. PUFAs in the diet have long been considered essential to the growth and proper nutrition of humans and other vertebrates. It was reported that atherosclerosis and thrombosis represent essential fatty acid deficiencies, but rather that the polyunsaturated fat may affect these pathological processes through other mechanisms. There is evidence from epidemiology that marine *n*-3 PUFAs are associated with a reduced risk of coronary heart disease. This was originally found in Greenland Eskimos with an extremely high intake of *n*-3 PUFA (10–14 g/day) and later also reported in several other populations (Schmidt *et al*., 2005; Kris-Etherton *et al*., 2002) including Western populations with an average intake of marine *n*-3 PUFA below 0.2–0.4 g/day (He *et al.*, 2004). Fish consumption was inversely related with fatal CHD and sudden cardiac death (He *et al.*, 2004).

2.2.4. Role of Fatty Acids to Combat Inflammatory Disorders

An increased intake of *n*-3 PUFA is associated with a reduced proinflammatory T cell response, including diminished proliferative capacity in response to mitogenic stimuli and impaired production of a critical T cell growth factor, IL-2. A short-term feeding paradigm in mice with diets enriched with fish oil or EPA and DHA (97% pure) ethyl esters was found to suppress inflammatory reactions and mitogen-induced proliferation of T cells (Fowler *et al*., 1993; Chapkin *et al.*, 2002). The loss of proliferative activity was accompanied by reduction in IL-2 secretion and IL-2R -chain mRNA transcription, suggesting that dietary EPA and DHA act, in part, by interrupting the autocrine IL-2 activation pathway. In addition, dietary EPA and DHA blunted the production of intracellular second messengers, including DAG and ceramide, following mitogen stimulation *ex vivo* (Fowler *et al.* 1993; Jolly *et al.*, 1997).

The incorporation of *n*-3 PUFA into T cell membrane phospholipids alters lipid rafts by reducing sphingomyelin levels and altering glycerophosphocholine (GPC) and glycerophosphoethanolamine (GPE) fatty acyl composition leading to the suppression of inflammatory T Cell Protein Kinase Cθ (PKCθ) into lipid rafts,

thereby down-regulating PKC-mediated signaling cascades to culminate inflammatory nuclear factor-kB (NF-kB), interleukin-2 (IL-2) secretion, and impaired lymphoproliferation and inflammation. It was proved that the dietary DHA, an effector molecule capable of attenuating immune-mediated inflammatory diseases (Meydani *et al*., 1991; Belluzzi *et al*. 1996), down-regulates the PKCθ signaling axis in murine T cells.

Recent studies have identified novel families of mediators formed from EPA and DHA, termed E- and D-series resolvins, respectively. These mediators are derived from *n*-3 LC-PUFAs *viz*., EPA and DHA produced via the cyclooxygenase and lipoxygenase reactions, and are considered to be anti-inflammatory (Calder, 2006). The identification of resolvins is an exciting new area of research in *n*-3 fatty acids and inflammation, and the implications to a variety of conditions may be of great importance. These mediators may explain many of the anti-inflammatory actions of *n*-3 fatty acids that have been described (Serhan *et al.* 2002). Arachidonic acid is metabolized by COX-1 and COX-2 to prostaglandins (PGG_2 and PGH_2). PGH_2 serves as a substrate for a series of downstream synthases to give rise to the prostaglandins. In contrast anti-inflammatory resolvins are biosynthesized from EPA (18R-HEPE series) and DHA (17R-HDHA series) (Fig. 2.5) leading to potent inhibitors of inflammatory mediator recruitment *in vitro* and *in vivo* (Serhan *et al*. 2002; Gilroy *et al.,* 2004). The *n*–6 and *n*–3 PUFAs may promote and suppress uncontrolled growth/cancerous growth of cells, respectively. In cancerous cells, COX-2, and 5-LOX are overexpressed, resulting in overproduction of *n*-6 fatty acid 20:4*n*–6- derived eicosanoids, which are the primary causal agents of inflammation. The recognition that the marine *n*-3 PUFAs have anti-inflammatory actions has led to the idea that supplementation of the diet of patients with inflammatory diseases may be of clinical benefit to those patients. Supplementation trials (usually with fish oils) have been conducted in a range of such diseases. The best explored have been rheumatoid arthritis, inflammatory bowel diseases (Crohn's disease and ulcerative colitis) and asthma, but there are trials in many other inflammatory diseases as well (Calder, 2006; Grimble *et al*., 2002). Marine *n*-3 PUFA have anti-inflammatory effects in patients with rheumatoid arthritis (Grimble *et al*., 2002). Many trials with fish oils have been conducted in this condition. Almost all of these trials show a benefit from fish oil, such as reduced duration of morning stiffness, reduced number

of tender or swollen joints, reduced joint pain, reduced time to fatigue, increased grip strength and decreased use of non-steroidal anti-inflammatory drugs (NSAIDs). It is important to note that very high doses of marine *n*-3 fatty acids have been used in most of these studies. Nevertheless, at these intakes, the evidence that marine *n*-3 PUFA are effective in rheumatoid arthritis is convincing.

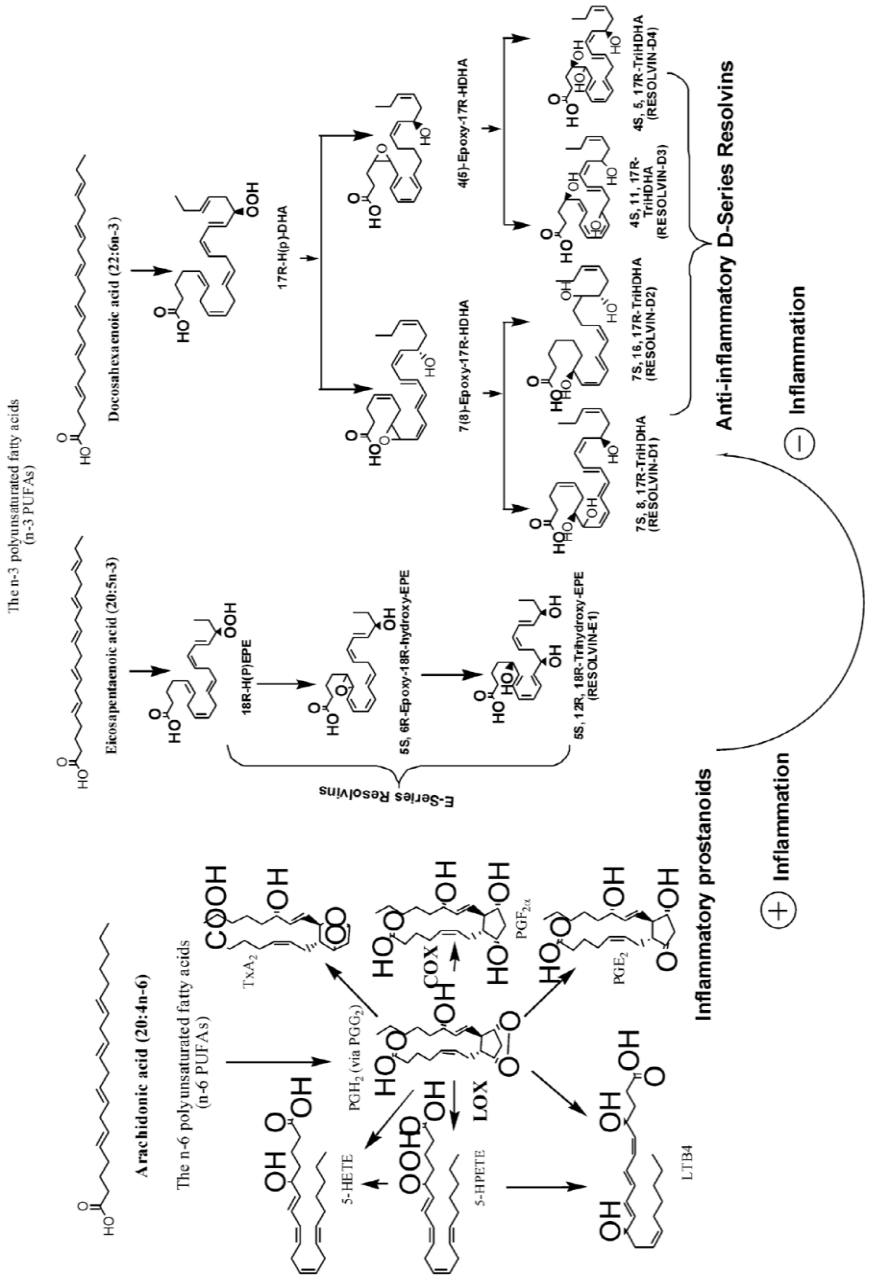


Fig. 2.5. Biosynthetic route of anti-inflammatory mediators from long chain *n*-3 PUFAs

2.3. General Overview of Fatty Acid Synthesis and Catabolism

Fatty acid synthesis is a metabolic process to combine eight C2 – moieties (-$CH_3C(=O)$ group from $CH_3COSCoA$) to synthesize saturated fatty acid with C16-moiety ($C_{16}H_{32}O_2$) , which thereafter modified to form homologous fatty acid analogues. These modifications include: elongase catalyzed chain elongation to synthesize fatty acids with longer hydrocarbon chain, e.g., stearic acid ($C_{18}H_{36}O_2$), arachidic acid ($C_{20}H_{40}O_2$), and so on. These SFAs, on desaturation yield unsaturated fatty acid analogues. In general, fatty acid synthesis takes place in cytoplasm of liver, adipose, central nervous system, and lactating mammary gland tissues of human. Glycolytic breakdown of glucose yields acetyl CoA through pyruvate ($CH_3COCOOH$) by aerobic glycolysis that is starting material for fatty acid synthesis. Acetyl CoA serves as substrate to synthesize citrate that transported out of mitochondria to cytosol and generates acetyl CoA. The overall process for fatty acid synthesis from acetyl CoA ($CH_3COSCoA$) is in Fig 2.6.

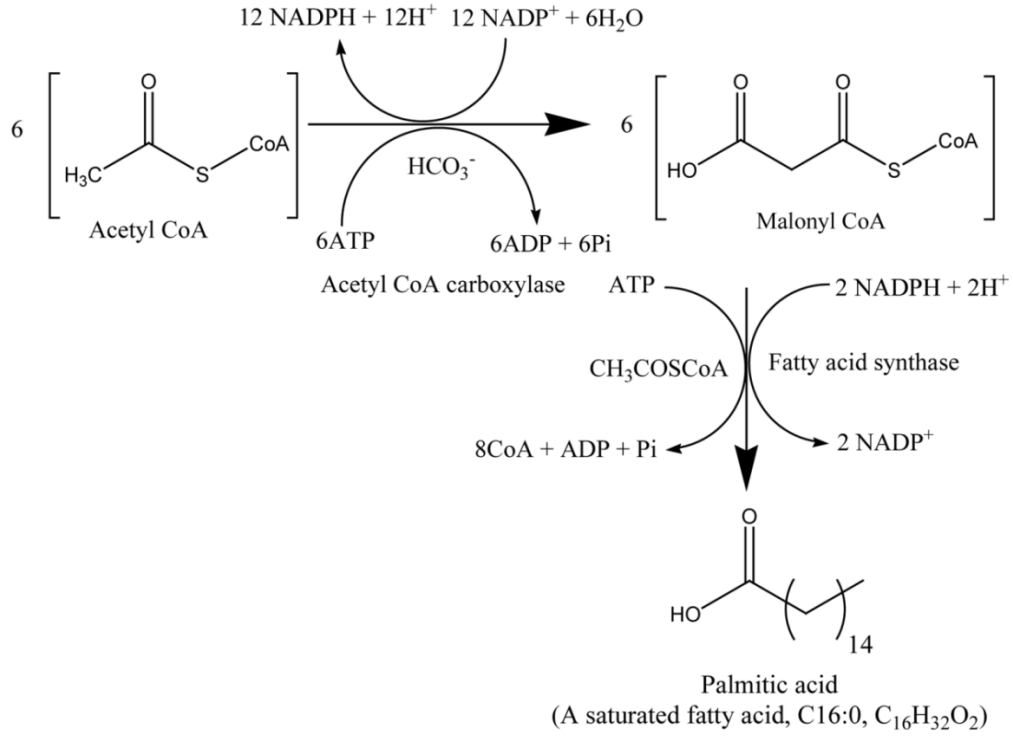


Fig. 2.6. The overall process for fatty acid synthesis from acetyl CoA ($CH_3COSCoA$)

Fatty acid synthesis takes place in cytosol using acetyl CoA as substrate. Acetyl CoA carboxylase catalyzes the following reaction to yield malonyl CoA and one molecule of acetyl CoA then interact sequentially with fatty acid synthase to yield the final product, palmitate. Thereafter the lower hydrocarbon chain fatty acids elongate in ER membrane surface using malonyl CoA as the source of the added carbons (Fig. 2.7). The fatty acids thus elongated were desaturated by a cascade of enzyme activities where a *cis* olefinic double bond is formed in the hydrocarbon chain. The desaturation reaction requires an electron transport system involving cytochrome Δ^5- desaturase , and NADPH cytochrome Δ^5- reductase. In human beings and other vertebrates there are four different desaturases, each with a different specificity viz., 9, 6, 5, 4 i.e., signifying that they act on 9-, 6-, 5- or 4-carbons. A minimum chain length of C16-18 moiety is necessary for desaturation process. The specificity of distance from -COOH carbon, along with the need for a C16-18 moiety signifies that *n*-6 and *n*-3 fatty acids (otherwise *n*-6 and *n*-3) are not synthesized in humans. However, these fatty acids are essential for several metabolic functions, and therefore must be supplied externally in the diet. For example, desaturation and C2 elongation result in conversion of 18:2*n*-6 to 20:4*n*-6. The overall reaction of anabolism of fatty acids to form unsaturated fatty acids is as follows (Fig. 2.7):

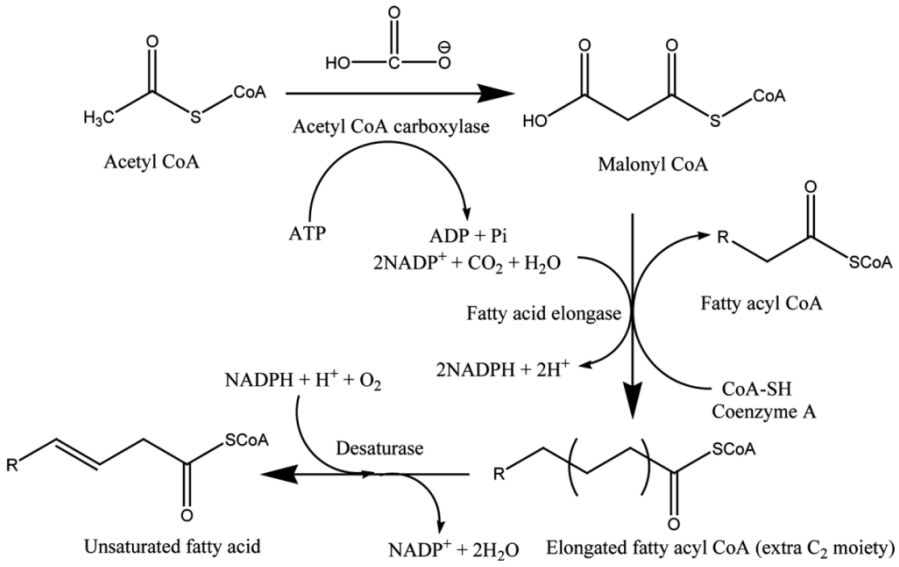


Fig. 2.7. Synthesis of unsaturated fatty acids from acetyl CoA

Fatty acids are stored in adipocytes as triacylglycerol that must be hydrolyzed to release free fatty acids. During catabolism, the fatty acids are activated by fatty acyl CoA synthetase. The forward reaction is accelerated by hydrolysis of PPi to Pi. Fatty acyl CoA thus formed is impermeable to the inner mitochondrial membrane, and therefore is carried in the form of fatty acyl carnitine. Fatty acyl CoA reacts with carnitine in a reaction catalyzed by carnitine acyltransferase I (CAT I), yielding CoA and fatty acylcarnitine. Carnitine acts as a carrier of "acyl" moiety through inner mitochondrial membrane. In inner mitochondrial surface fatty acyl carnitine yields fatty acyl CoA and carnitine in a reaction catalyzed by carnitine acyltransferase II (CAT II). The fatty acyl CoA thus formed undergoes β-oxidation in a process by that long chain fatty acyl CoA undergoes degradation to yield acetyl CoA. Four individual reactions take place in sequence in β-oxidation, each catalyzed by a separate enzyme to yield acetyl CoA. The steps are: dehydrogenation between the α and β-carbons (C2 and C3) in a FAD-linked reaction, hydration of the double bond by enoyl CoA hydratase, a second dehydrogenation in a NAD-linked reaction, and thiolytic cleavage of the thioester by beta-ketoacyl CoA thiolase (Fig. 2.8).

There are three fatty acyl CoA dehydrogenases. Each is specific for a different acyl chain length, so different enzymes are involved in different stages of β-oxidation. Long chain fatty acyl CoA dehydrogenase acts on chains greater than C12. Medium chain fatty acyl CoA dehydrogenase acts on chains of C6-12. Short chain fatty acyl CoA dehydrogenase acts on chains of C4-6. The products are acetyl CoA and a long chain fatty acyl CoA that is C2 shorter than the original fatty acyl CoA. The shortened fatty acyl group is now ready for another round of β-oxidation. The summery of complete oxidation of an odd-chain fatty acid is as follows:

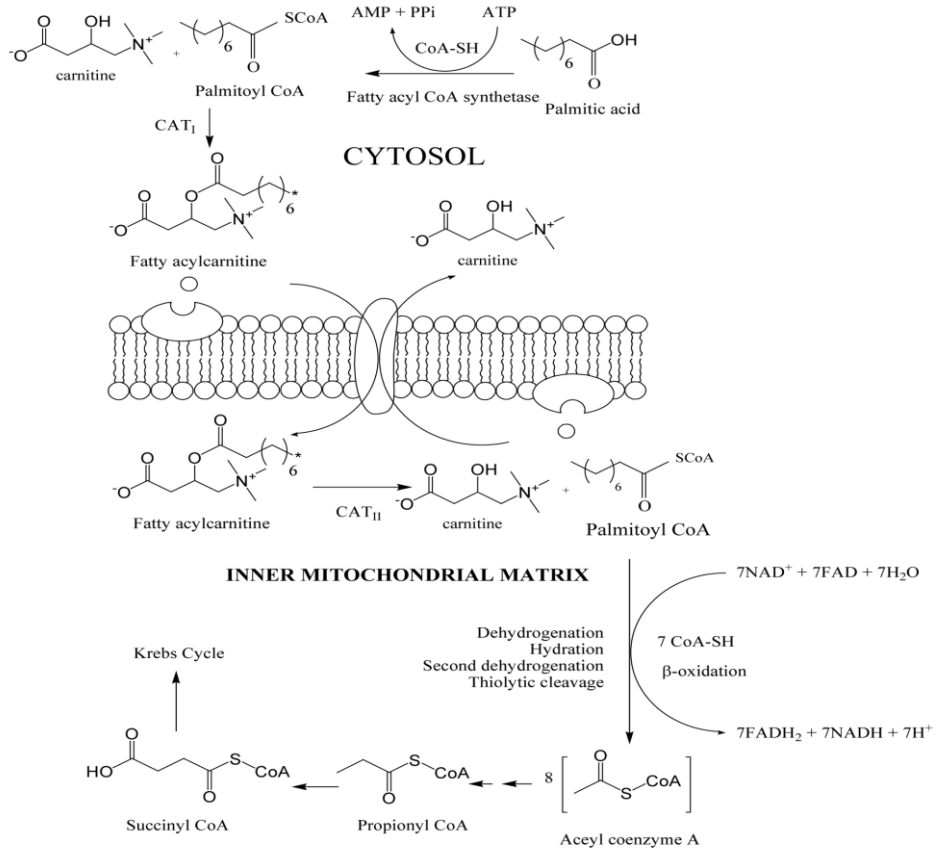


Fig. 2.8. The β-oxidation of palmitoyl CoA to form acetyl CoA. This diagram shows production of propionyl CoA from an odd-chain fatty acid and the subsequent conversion of propionyl CoA to succinyl CoA, which can be metabolized through Krebs cycle

2.4. Metabolic Pathway of *n*-3 Fatty Acids

The metabolic pathway for *n*-3 oils is well defined. *n*-3 oils within this metabolic pathway range from 18 to 24 carbons long, and vary from 3 to 6 carbon-carbon unsaturated double bonds. The ALA is an 18-carbon fatty acid that can be converted to longer chain and more unsaturated fatty acids via a series of elongation and desaturation steps. Humans actually convert little of ingested ALA to EPA because of limited delta-6 desaturase activity (Fig. 2.9) (the first biochemical step in the pathway) (Chilton *et al.*, 2008). Whole body conversion of ALA through the pathway to DHA is less than 5% in humans. Oxidation of dietary ALA to CO_2 accounts for about 25% of ALA in the first 24 hours and may reach 60% by 7 days (Brenna *et al.*, 2009). To the degree that Δ^6-desaturase does function, it converts ALA to stearidonic acid (SDA) by desaturation of an additional carbon-carbon bond. Both dietary and

biosynthesized SDA may be metabolized to EPA and docosapentaenoic acid (DPA) by the actions of elongase and Δ^6-desaturase with relative ease compared to further metabolism to DHA that depends on Δ^6-desaturase activity followed by beta-oxidation (James *et al.*, 2003). There is some evidence that the *n*-3 pathway is affected by gender based differences in hormone levels.

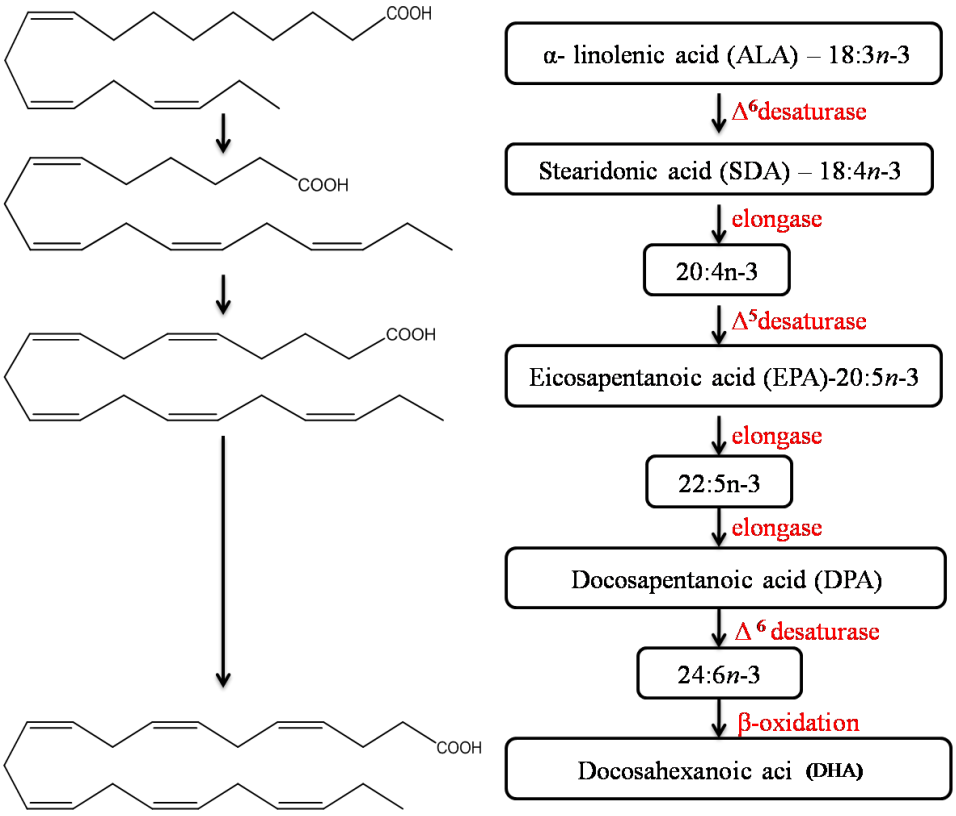


Fig. 2.9. Metabolic pathway of *n*-3 fatty acids forming EPA and DHA from the precursor ALA

2.5. Extraction and Refining of Fish Oils

On a global scale fish body oils make up the majority of the marine oils produced with a small amount of oil coming from fish and shark livers, krill, squid, marine mammals, marine and freshwater algae, and yeasts. According to the Food and Agriculture Organization (FAO) of the UN (FAO 1986), raw material used for the production of fishmeal and fish oil falls into several categories:

1. Fish caught specifically for reduction to fishmeal and fish oil such as menhaden, anchovy, capelin and sardines.
2. Incidental or by catch from another fishery
3. Fish by-products from the edible fisheries such as cuttings from filleting operations, fish cannery waste, roe fishery waste and more recently surimi processing waste.

Fig. 2.10. Photographs showing the marine fishes in the landing centre of Southwest coast of India

Several methods can be used to extract fish oils from different raw materials but these methods vary widely in terms of yield and quality of the extracted oils. The production of fish oil deals with the separation of lipids from other constituents of the fish. Extraction of the crude fat/oils are done using three main ways, namely rendering (wet/dry), mechanical pressing and using a solvent extraction (Kiple & Ornelas 2000). Rendering method is the oldest method used by humans, and often referred to as a traditional method. Among the three types of extraction that are commonly used, rendering is the earliest method used by humans. The main principle of this method, either wet or dry is by disrupting the tissue of the material by applying heat to allow oil separation. Dry rendering is done by heating a material so that the fat melts out and can be separated (Mc-Williams 2001). Wet rendering interim of aqueous extraction, containing three important processes; material crushing, cooking process - which at first development is using heated water- and oil separation either using a pressing or centrifuging (Kiple & Ornelas 2000). Another method used industrially in obtaining fish oil is hydraulic pressing; a batch process whereby the oil is obtained or expressed by hydraulic pressing a mass of moderately cooked oil-bearing fish. Large-scale oil development started when a mechanical pressing method was applied, and reached its peak 120 years ago when solvents were initially introduced for oil extraction (Matthäus 2008). The extraction of oil using a full-press, especially with the cold-pressed method is reblooming. Solvent extraction is among the most commonly used methods of isolating lipids from food samples (including fish) and of determining the total lipid content of foods. The principle is based on the fact that lipids are soluble in organic solvents, but insoluble in water, hence providing a convenient method of separating the lipid components in the food samples from water-soluble components such as protein, carbohydrates, minerals, and water itself (McClements 2003). Soxhlet extraction is another commonly used method for determination of total lipids in dried samples. The main disadvantages of the soxhlet extraction technique are that: a relatively dry sample is needed (to allow the solvent to penetrate), it is destructive, and it is time-consuming (McClements 2003). Other methods that are used to extract valuable *n*-3 PUFAs using lipases or proteases hydrolysis (Linder *et al*., 2005). Recently, supercritical fluid extraction (SFE) has become an attractive technology for obtaining high quality fish oil

from some by-products (Rubio-Rodríguez *et al.,* 2010). At this point, it is necessary to emphasize that usually, the final product of any extraction process is unrefined oil that contains non-triglycerides and other odor components, being therefore most often necessary to include a refining process before obtaining an edible fish oil.

The chemical refining method involves distinct stages such as degumming (to separate phospolipids), neutralizing (to clear free fatty acid and decrease oil acidity), bleaching (to adsorb contaminants and pigment) and deodorization (to remove smelly compounds). Degumming is only a partial refining, since free fatty acids are not reduced and even the gums are not completely removed. Degumming is the processes by which phosphatides and certain other mucilaginous materials are removed from the fish oil by treatment with 2-3% water or with aqueous solutions of boric acid or salts such as $NaCl/H_3PO_4$ at 30 - 50 ^oC. The oils are processed by chemical or alkaline refining steps including degumming to remove phospholipids and other gummy materials, neutralization with caustic soda to remove free fatty acids (FFA), washing with water to remove extra alkali, bleaching to remove soap and trace metals, and deodorization by vacuum distillation to remove residual FFA, aldehydes, ketones, alcohols and other compounds. After the degumming treatment, the oil is treated with NaOH/KOH to neutralize the free fatty acid content and to solubilize phospholipids, N_2 and sulphur containing compounds and some pigments. In the way a large part of the impurities can be removed in the aqueous phase resulting from the first centrifugation step. The oil is then washed with water to remove soap and centrifuged before drying. The main objective of oil bleaching is to reduce colored materials and natural pigments and to absorb suspended mucilaginous, colloid like matters and any trace of soap if still present (Chang et al., 1992). The process leads to fish oil product with improved color, flavor and oxidative stability, free from impurities (Bimbo 1989). The process involves heating the oil to 90-110 ^0C, often under vacuum condition, in the presence of bleaching clay by weight depending on oil quality, for the desired period of time and then removed of the spent day by filtration (Bimbo 1989; Young 1982). Deodorization is a mass-transfer purification process of the oil industry that aims towards vaporizing odoriferous compounds and free fatty acids from the oil by applying high temperatures and low pressures. Volatile flavor compounds are removed principally by a vacuum steam

deodorization step, and conventional processing employs temperatures in the range of 200 - 275 ^{o}C for vegetable oils (Zehnder 1976) and ~190-230 for fish oils (Chang 1967). Apparently these conditions generally remove off-flavors; such elevated temperatures cause formation of artifacts through isomerization and polymerization reactions in fish oils and other oils containing PUFAs (Karahadian & Lindsay 1990). The oxidation products of the PUFAs of fish oils, whether free or bound in the TAGs are generally regarded as the causative agents of characteristic fish odor of fish oils.

2.6. Purification of Fish Oils

The technologies available for purifying individual PUFAs and PUFA concentrates from fish oil are based on differences in physicochemical properties associated with the number of double bonds in the molecule or the chain length. A survey of the literature revealed a diverse range of methodologies for enrichment and purification of LC-PUFAs. These included urea inclusion complexation, molecular distillation, iodolactonization, low temperature fractional crystallization, salt solubility methods, and liquid–liquid extraction-fractionation using aqueous silver nitrate solution (Guil-Guerrero *et al*., 2007; Lopez-Martinez *et al*., 2004; Mjøs, 2008). Several chromatographic techniques such as counter-current chromatography, reverse phase chromatography (C_{18}), silver-ion impregnated silica/alumina chromatography have also been used for separation and purification of individual LC-PUFAs (Sahena *et al*., 2009). Argentation silica gel column chromatography has been employed to obtain high purity EPA methyl ester from hydrolysates of fish oil that contained other PUFA esters including linolenic acid and oleic acid (Guil-Guerrero *et al*., 2003). PUFAs have been purified from the red microalga *Porphyridium cruentum* by the urea inclusion method followed by silica gel column chromatography of the urea concentrate (Guil-Guerrero *et al*., 2000). EPA and DHA have been concentrated from *Isurus oxyrinchus* (Guil-Guerrero *et al*., 2007). Solvent winterization of seed oil and free fatty acids was employed to obtain γ-linolenic acid concentrates from seed oils of *Echium fastuosum* and *Borago officinalis* (Lopez-Martinez *et al*., 2004). There are reports to concentrate PUFAs from cod liver oil and edible oils (Guil-Guerrero *et al*., 2001; Guil-Guerrero *et al*., 2003). A polyethylene

glycol stationary phase was evaluated for the separation of EPA and DHA methyl esters (Mjøs *et al*., 2008). Mackerel processing waste was used for concentrating PUFA using chloroform:methanol (2:1) and *n*-hexane:isopropanol (3:2) (Zuta *et al*., 2003). Fractionations of EPA and DHA were reported to be carried out by supercritical fluid extractions (Sahena *et al*., 2009).

The use of enzymes in industrial processes is fairly recent but it has become a good alternative to traditional methods since it can be simpler and cheaper regarding investment cost, energy expenses and processing. These advantages have promoted the research on the use of enzymatic technology to release lipids from fish avoiding the use of solvents and high temperatures. Liaset *et al*. (2003) studied the enzymatic hydrolysis of salmon frames with proteases and the composition of the different fractions obtained after separation by centrifugation. They reported that this process enables to obtain *n*-3 enriched oil with a good recovery (about 77%) as well as several interesting products such as peptides or essential amino acids. Linder *et al.* (2005) developed another enzymatic method to extract oil from ground salmon heads at middle temperature (55 °C) using different commercial enzymes: a protease (alcalase), an exopeptidase (neutrase) and an endo-peptidase (flavourzyme). They concluded that the highest oil recovery (17.4% after 2 h) was obtained by using alcalase, being close to the recovery obtained by the Bligh and Dyer method (20%). Earlier reports studied the influence of the composition and state of the substrate on the yield and composition of the hydrolysis products (oil fraction, hydrolysed protein, sludge and emulsion) (Šližyte *et al.,* 2005) and on the chemical composition of the lipid phase (Daukšas *et al.,* 2005). The same authors also studied the influence of initial heating of the raw material on the yield and the chemical composition of the oil extracted by enzymatic hydrolysis of cod by-products using alcalase and lecitase (Šližyte *et al.,* 2005). Liu et al. (2006) proposed an enzymatic process based on a proteolytic extraction of oil from crude tuna heads followed by a urea complexation step, obtaining a mixture of DHA and EPA with a purity of 85.02% and a yield of 25.10%. The general conclusion reached by the authors that have studied fish oil extraction by using enzyme technology is that this technology yields better oil than thermal treatments.

2.7. Oxidation of Fish Oils

2.7.1. Chemistry of Lipid Oxidation

The LC-PUFAs are highly susceptible to oxidative degradation during processing, handling and storage. This oxidation of fish oil leading to undesirable off-flavors and loss of nutritional value. Unsaturated fatty acids are prone to photo-oxidation during light exposure, enzymatic oxidation when exposed to lipooxygenases, and auto oxidation, which is the direct reaction of molecular oxygen with organic compounds (Frankel 2005). The degree and rate of lipid oxidation is influenced by the composition of fatty acids, oxygen concentration present, temperature, surface area, water activity and presence of anti and pro oxidants (Fennema *et al.,* 2007). Photo-oxidation is generally of little concern, as light absorption cannot affect the lipids unless they are exposed to direct sunlight or fluorescent light without suitable protection (List *et al.,* 2005). Enzymatic oxidation is also of less concern in oils, because lipooxygenases are inactivated by heating during refining (Fennema *et al.,* 2007). Based on these facts, auto oxidation is the primary concern of oxidation in refined oils.

The susceptibility to fatty acid radical formation increases with degree of unsaturation. The dissociation energy in a carbon-hydrogen bond associated with an electron-rich double bond is lowered by 9 kcal mol^{-1}, compared to a saturated carbon-hydrogen covalent bond. The lowered dissociation energy makes unsaturated carbon chains more susceptible to hydrogen abstraction (Fennema *et al.,* 2007). The process of lipid oxidation can be described in three steps; initiation, propagation and termination.

Initiation

In the case of PUFAs, conjugated double bonds are rapidly formed upon the abstraction of hydrogen in the initiation step (Fennema *et al.,* 2007). When a hydrogen atom (H) is abstracted from an unsaturated fatty acid (RH) forming an alkyl radical ($R^{\cdot}$), lipid oxidation is initiated. The initiation process is summarized in the following equation (Chaiyasit *et al.,* 2007).

$$\mathbf{RH \rightarrow R^{\cdot} + H^{\cdot}}$$

Generation of this lipid radical ($R^{\cdot}$) is thermodynamically unfavorable and is usually initiated by the presence of other radical compounds (R), singlet state oxygen ($^1O_2$), decomposition of hydroperoxides (ROOH), or pigments that act as photosensitizers. Thus the oxidation process proceeds to the propagation step as the alkyl radical reacts with oxygen.

Propagation

In order to stabilize, the alkyl radical ($R^{\cdot}$) usually undergoes a shift in the position of the double bond (*cis* to *trans*) and production of a conjugated diene system. The $R^{\cdot}$ can react with O_2 to form a high-energy peroxyl radical ($ROO^{\cdot}$) in the propagation step. The propagation process is summarized in the following equation (Chaiyasit *et al.*, 2007).

$$\mathbf{R^{\cdot} + O_2 \rightarrow ROO^{\cdot}}$$

The peroxyl radical can then abstract a hydrogen atom (H) from another unsaturated fatty acid (RH) forming a lipid hydroperoxide (ROOH) and a new, free alkyl radical ($R^{\cdot}$) as follows:

$$\mathbf{ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot}}$$

These lipid hydroperoxides (ROOH) are the primary products of lipid oxidation. They are tasteless and odorless; however, in the presence of heat, metal ions, and/or light, they can decompose to compounds that contribute off-odors and off tastes. Alkoxy radicals ($RO^{\cdot}$) can also abstract H from unsaturated fatty acids continuing the chain reaction

Termination

In the termination step two radicals are joined to form a non-reactive unit. During conditions of oxygen excess, peroxyl radicals ($ROO^{\cdot}$) will join to make the termination product. This is a result of oxygen being added to the alkyl radical and leaving peroxyl as the main radical in the reaction. The termination step is given in the following equations (Chaiyasit *et al.*, 2007):

$$\mathbf{ROO^{\cdot} + R^{\cdot} \rightarrow ROOR}$$

In conditions of low oxygen levels, the termination products are a result of interference between alkyl radicals, creating fatty acid dimers (Fennema *et al.*, 2007).

$$\mathbf{R^{\cdot} + R^{\cdot} \rightarrow RR}$$

In lipid oxidation the oxidation process generally show a lag phase followed by an exponential increase in oxidation rate. During the lag phase the oxidation is relatively slow and at a steady rate. Once the exponential phase is reached, fatty acid decomposition products quickly form. Hydroperoxides are the main primary oxidation products, accumulating during the initiation and propagation step of the oxidation process (Fennema *et al.*, 2007). After the maximum hydroperoxide level has been reached, a drop in hydroperoxides will be seen as the hydroperoxides decompose into a variety of secondary oxidation products (Frankel 2005). The drop in hydroperoxides is observed when the rate of decomposition into secondary products exceeds the formation rate (Shahidi & Wanasundara 2002). Theoretically, this symbolizes that the primary oxidation products will dominate in the early stage and secondary oxidation products will dominate in later stages of the oxidation process.

The hydroperoxides themselves do not contribute to the off-aromas causing rancidity (Reindl & Stan 1982). The hydroperoxide will initially decompose into an alkoxyl radical ($RO^{\cdot}$). The formation of the high energy alkoxyl radical is the starting point for the cleaving of the aliphatic chain in fatty acids, known as the β-scission reaction (Frankel 1998). The cleaving of the aliphatic chain produces aldehydes in addition to a radical on the aliphatic chain. This radical (e.g. alkyl radical) can theoretically react further with hydrogen radical to form a hydrocarbon, a hydroxyl radical to form an alcohol or oxygen to produce a hydroperoxide. The mentioned alkoxyl radical can convert to a ketone by loosening an electron, or an epoxide through bonding to adjacent carbons. Some of the decomposition products contain intact pentadiene systems. The presence of these double bonds can result in

formation of decomposition products because of further hydrogen abstraction or reaction with $^1O_2$ (Fennema *et al.*, 2007).

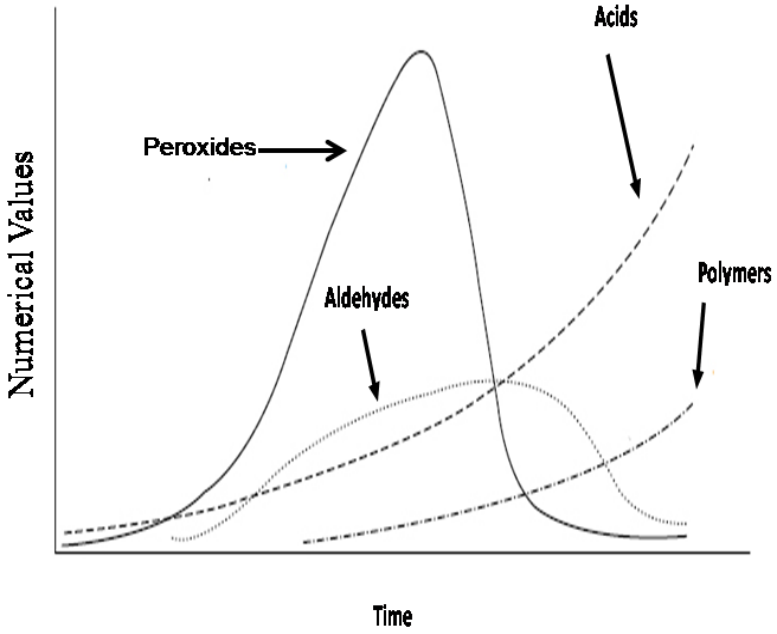


Fig. 2.11. Time relationship among peroxides and their degradation products after oxidation of marine oils

Free fatty acids are electron-deficient at the oxygen atom of the carbonyl group (C=O); unsaturated fatty acids are also electron-deficient at points of carbon–carbon unsaturation (C=C). These electron deficient regions make fatty acids susceptible to attack by a variety of oxidizing and high-energy agents generating free radicals (Nawar & Fennema 1996). Triglycerides contain straight chains of primarily 16- to 18-carbon fatty acids and minimal amounts of unsaturated fatty acids. Phospholipids in tissue membranes contain up to 15 times the amount of unsaturated fatty acids (C18:4, C20:4, C20:5, C22:5, and C22:6) found in triglycerides. They are much more susceptible to oxidation because of the increase in the number of points of carbon–carbon unsaturation (C=C) (Elmore *et al.*, 1999).

2.7.2. Negative Effects of Lipid Oxidation of *n*-3 PUFAs

Lipid oxidation of fish oil will give rise to the formation of free radicals, lipid hydroperoxides and secondary oxidation products (aldehydes, ketones, alcohols, hydrocarbons) (Fig. 2.12).

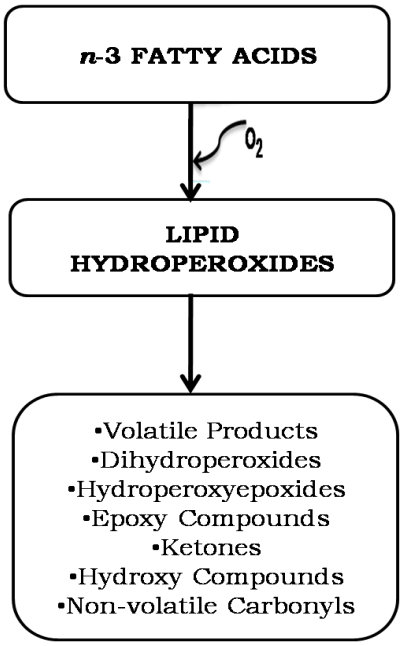


Fig. 2.12. Secondary oxidation products from decomposition of lipid hydroperoxide

The secondary oxidation products are responsible for the undesirable changes in the aroma and flavour properties of foods. Among these compounds the vinyl ketones and the *trans*, *cis*-alkadienals have the lowest flavour thresholds in oils .In contrast, hydrocarbons (alkanes and alkenes) have the highest flavour thresholds (Frankel 2005). Due to high unsaturation in EPA and DHA, oxidation processes may lead to a complex mixture of hydroperoxides and a myriad of volatile, non-volatile and polymeric secondary oxidation products. Secondary oxidation products such as aldehydes, ketones, alcohols and hydrocarbons are associated not only with negative effects on the odor, flavor, color and texture but also loss of nutritional qualities such as amino acids, vitamins, polyphenols, phytosterols and squalene (Ringseis & Eder 2010). Consumption of oxidized edible oil can lead to toxicity and biological problems such as diarrhea increase in depression and tissue or organ damage due to the oxidation products from unsaturated fatty acids. Liver damage was observed in rats that were fed by oxidized oil whereas liver damage was not developed in the rats fed by fresh oil (Totani *et al.*, 2008).

2.7.3. Measurements of Lipid Oxidation

The lipid oxidation may be assessed in several ways, among which changes in the initial reactants and formation of oxidation products are most commonly assessed. The common analytical tools to assay the lipid oxidation are detailed as follows:

2.7. 3.1. Weight Gain Method

The consumption of oxygen during the initial stage of autoxidation results in an increase in the weight of fat or oil, which theoretically reflects its oxidation level. Heating of the oil and periodically monitoring for weight gain is a traditional method for evaluating oxidative stability.

2.7.3.2. Measurement of Reactant Change

Lipid oxidation can also be assessed by quantitatively measuring the loss of initial substrates. In foods containing fats or oils, unsaturated fatty acids are the main reactants whose composition changes significantly during oxidation. Changes in fatty acid composition provide an indirect measure of the extent of lipid oxidation (Melton 1983).

2.7.3.3. Determination of Primary Oxidation Products

Lipid oxidation involves the continuous formation of hydroperoxides as primary oxidation products that may break down to a variety of nonvolatile and volatile secondary products (Melton 1983). UV measurement of conjugated dienes and trienes are one way to detect primary oxidation products. The peroxide value (P.V.) represents the total hydroperoxide content and is one of the most common quality indicators of fats and oils during production and storage (Antolovich *et al.*, 2002; Riuz *et al.,* 2001). A number of methods have been developed for determination of the P.V., among which the iodometric titration, ferric ion complex measurement spectrophotometry, and infrared spectroscopy are most frequently used (Yildiz *et al.*, 2003).

2.7.3.4. Determination of Secondary Oxidation Products

The primary oxidation products (hydroperoxides) are unstable and susceptible to decomposition. A complex mixture of volatile, nonvolatile, and polymeric secondary oxidation products is formed through decomposition reactions, providing various indices of lipid oxidation. Secondary oxidation products include aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids, and epoxy compounds, among others. The analysis for determination of secondary oxidation products therefore tends to focus on a single compound or group of compounds.

The p-anisidine value (pA.V.) is based on the reaction between p-anisidine and aldehyde compounds present in oil samples at acidic conditions. The reaction produces a yellow colored compound with absorbance at 350 nm (Frankel 2005). The reaction does not include use of any strong acids or high temperature and therefore an advantage of the method is minimized influence on hydroperoxide decomposition.

Holm suggested a combined expression of peroxides and secondary oxidation products, and therefore developed the concept TOTOX value. Holm demonstrated that an increase of one P.V. unit corresponded to increase in two pA.V. units. Together this established the TOTOX value = 2P.V.+ pA.V., giving a value of the total oxidation status in oil (Holm 1972). The total oxidation can be used to measure the progression of deterioration that occurs in oil and provide information on the formation of primary and secondary oxidation products (Hamilton & Rossell 1986).

The thiobarbituric acid reactive species (TBARS) test was proposed over 40 years ago and is now one of the most extensively used methods to detect oxidative deterioration of fat-containing foods (Kishida *et al.,* 1993). During lipid oxidation, malonaldehyde (MA), a minor component of fatty acids with 3 or more double bonds, is formed as a result of the degradation of PUFAs. It is usually used as an indicator of the lipid oxidation process for the early appearance as oxidation occurs (Cesa 2004).

The oil stability index (OSI) method measures the formation of volatile acids by monitoring the change in electrical conductivity when effluent from oxidizing oils is passed through water (Gordon & Hudson 2001). During lipid oxidation, volatile

organic acids, mainly formic acid and acetic acid, are produced as secondary volatile oxidation products at high temperatures, simultaneously with hydroperoxides (Kiritsakis *et al.,* 2002; Schwarz *et al.,* 2001). In addition, other secondary products, including alcohols and carbonyl compounds, can be further oxidized to carboxylic acids (Kiritsakis *et al.,* 2002). The OSI value is defined as the point of maximal change of the rate of oxidation, attributed to the increase of conductivity by the formation of volatile organic acids during lipid oxidation (Miura *et al.,* 2002). The commercially available automated techniques for OSI assays are the rancimat (Metrohm Ltd.) and the oxidative stability instrument (Omnion Inc.). Rancimat is a rapid automated method, which agrees well with the active oxygen method (Presa-Owens *et al.,* 1995). In the rancimat assay, a flow of air is bubbled through heated oil, usually at 100 ^{o}C or above. For marine oils, temperatures as low as 80 ^{o}C are often used. Volatile compounds formed during accelerated oxidation are collected in distilled water, increasing the water conductivity. The conductivity change is plotted automatically and the induction period (induction time) of the oil or the time taken to reach a fixed level of conductivity is recorded (Kiritsakis *et al.,* 2000). The rancimat assay enables continuous monitoring of the oxidation process.

2.7.4. Use of Antioxidants to Control Lipid Oxidation

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. Free radicals are highly reactive, they are created when an atom or a molecule (a chemical that has two or more atoms) either gains or losses an electron (a small negatively charged particle found in atoms). Antioxidants are chemicals that interact with and neutralize free radicals, thus preventing them from causing damage. Antioxidants are also known as “free radical scavengers” (Antolovic *et al.,* 2002)

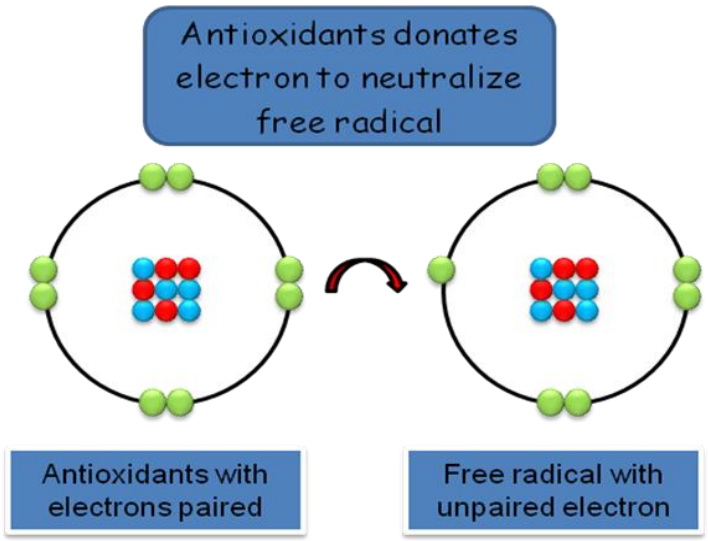


Fig.2.13. Antioxidant neutralizing free radical

Generally, antioxidants can be classified as natural and synthetic antioxidants. Based on their functions, antioxidants are further classified as primary or chain breaking antioxidants and synergists or secondary antioxidants (Shui & Leong 2004).

Primary antioxidants, also referred to as chain-breaking antioxidants, are characterized by their ability to react directly with free radicals, such as lipid, alkoxyl and peroxyl radicals, and convert them to more stable, non radical products. They are effective at extremely low concentrations of 0.01% or less and for some of them the effectiveness decreases as concentration is increased. At high concentrations they may become pro-oxidant due to their involvement in the initiation reactions (Bartosz & Druga 1995). They interfere directly with the free radical propagation process and they block the chain reaction. The reaction mechanisms of a primary antioxidant, AH (Antunes *et al.*, 1999) and secondary antioxidant BH, is shown in Fig. 2.14.

[1] Reaction of primary antioxidant, AH with lipid radical.

$$\mathbf{AH + ROO \rightarrow ROOH + A^{\cdot}} \quad \textbf{(a)}$$

$$\mathbf{RH + A^{\cdot} \rightarrow AH + R^{\cdot}} \quad \textbf{(b)}$$

$$\mathbf{AH + ROO^{\cdot} \rightarrow [ROO^{\cdot}AH]\ complex} \quad \textbf{(c)}$$

[2] Termination reaction

$$\mathbf{[ROO^{\cdot}AH]\ complex \rightarrow\ Non\text{-}radical\ Product} \quad \textbf{(d)}$$

$$A^{\cdot} + A^{\cdot} \rightarrow AA \quad \textbf{(e)}$$

$$A^{\cdot} + R^{\cdot} \rightarrow RA \quad \textbf{(f)}$$

$$A^{\cdot} + ROO^{\cdot} \rightarrow ROOA \quad \textbf{(g)}$$

[3] Regeneration of primary antioxidant

$$A^{\cdot} + BH \rightarrow AH + B^{\cdot} \quad \textbf{(h)}$$

Fig.**2.14.** Reaction mechanism of primary antioxidant with free radical. AH,antioxidant; ROO., lipid peroxyl radical; ROOH, lipid hydroperoxide; A·, antioxidant free radical; RH, unsaturated lipid; R., lipid radical; ROO. AH, stable compound (non-radical product); BH, secondary hydrogen donor; B·, secondary antioxidant free radical. The inhibitory reactions (A) to (a) influence the overall inhibition rate, and reaction (a) is more important than others. The stable resonance hybrid of antioxidant free radical A·, and the non-radical reaction (d) to (g) products thus produced are capable of inhibition the propagation of the chain reactions.

Secondary antioxidants or synergist delay the oxidative process by reacting with pro oxidants or oxidation intermediates. It can inhibit lipid oxidation by several different mechanisms including chelation of transition metals, oxygen scavenging, synergism between antioxidants and singlet oxygen quenching. The chelators are referred to as synergists since they greatly enhance the action of antioxidants. These transition metals promote lipid oxidation by reacting with hydroperoxides and thereby creating free radicals.

2.7.4.1. Synthetic Antioxidants

Synthetic antioxidants are chemically synthesized, since they do not occur in nature and are added to food as preservatives to help prevent lipid oxidation. These antioxidants fall into two major categories depending on their mode of action. Primary antioxidants and secondary antioxidants.

The primary antioxidants, which prevent the formation of free radicals during oxidation, can further include three major categories.

1) Free radical terminators
2) Chelating agents.
3) Oxygen scavengers

The radical terminators constitute the bulk of the synthetic antioxidants used as preservatives in food and these antioxidants prevent lipid oxidation by terminating the Free radical chains. The important examples of radical terminators include BHA,

BHT, tBHQ, and gallates such as propyl gallate, dodecyl gallate and octyl gallate (Fig. 2.15). The examples of oxygen scavengers which function as reducing agents, include sulphites, glucose oxidase and ascorbyl palmitate. The chelating agents such as, polyphosphatases and ethylenediaminetetraacetic acid (EDTA) prevent oxidation of lipids by binding the lipid oxidation catalysts such as heavy metals (iron, copper, etc). They do so by either precipitating the metal or by occupying all its coordination sites (Benzie *et al.*,2003).

Secondary antioxidants function by breaking down hydroperoxides formed during lipid oxidation into stable end products. Thiodipropionic acid and dilauryl theodipropionate are examples of secondary antioxidants (Daniel *et al.*,1986)

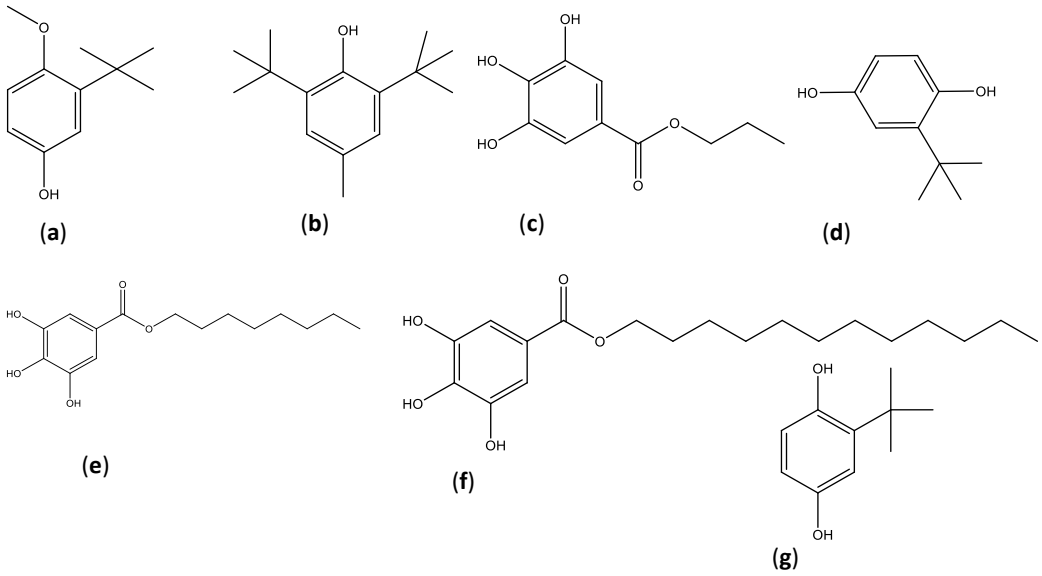


Fig.2.15. Chemical structure of synthetic antioxidants. a)BHT (b)BHA (c)PG (d)OG (e)DG (f) EDTA (g)TBHQ

Radical terminators, the major class of synthetic antioxidants, fall under a group of chemical compounds called phenols. Their specific mechanism of action involves transfer of the hydrogen of the phenolic hydroxyl group to a lipid free radical in order to form stable free radicals, which do not initiate or propagate the oxidation chain reactions (Fig. 2.16).

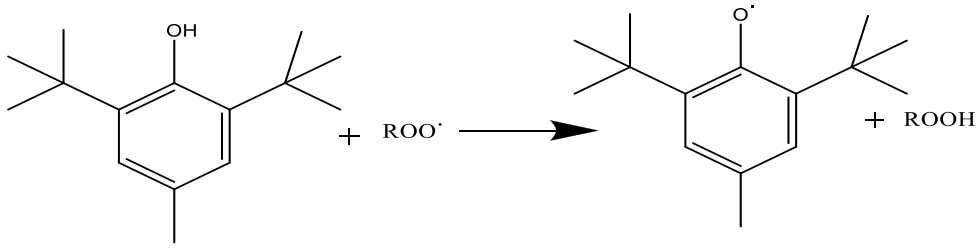


Fig. 2.16. Antioxidant mechanism of a radical terminator via the donation of an electron from a phenolic hydroxyl group

These reactions involving the donation of hydrogen to the lipid free radicals are highly exothermic and compete with the chain propagation reactions during the lipid auto-oxidation (Iverson *et al*., 1995)

Phenolic antioxidants such as BHA, BHT, tBHQ and propyl gallate all act as excellent antioxidants due to two main reasons. Firstly, their hydrogen bond dissociation energy is low making them efficient hydrogen or electron donors. Secondly, the phenoxy radicals generated after the donation of electrons to the lipid free radicals do not themselves generate other free radicals. They are relatively stable due to resonance stabilization by delocalization of electrons across the aromatic ring and the lack of suitable sites for attack by molecular oxygen (Fig. 2.17).

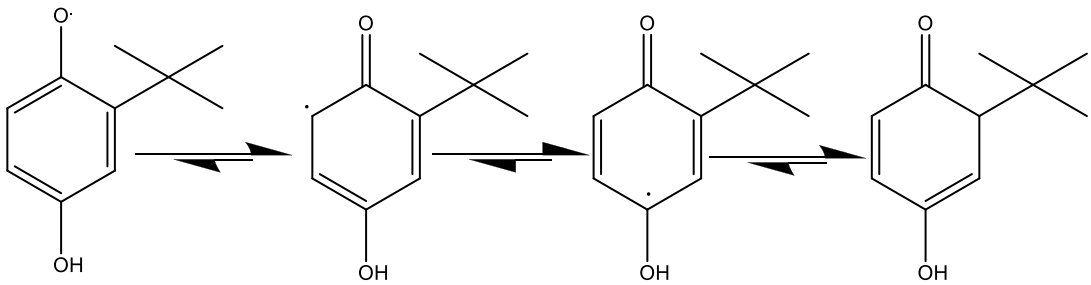


Fig. 2.17. Resonance stabilization of a phenolic antioxidant

Furthermore, the efficiency of these phenolic antioxidants varies depending on the absence or presence of certain other groups on the aromatic ring, mainly at the *ortho* and *para* positions. For example, the stability of the phenoxy radical is increased by the presence of bulky groups at the *ortho* position as in BHA, which is 2,6-di-tertiary-butyl,4-methoxyphenol (Fig. 2.18).

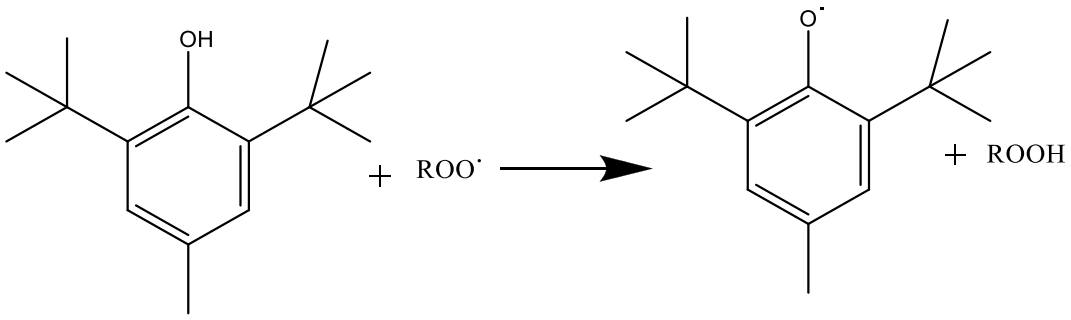


Fig. 2.18. Oxidation of BHA via donation of an electron from a phenolic hydroxyl group

The presence of bulky group introduces steric hindrance in the region of radicals, decreasing the rate of further propagation reactions. Another example, which illustrates the increase in antioxidant activity, is the presence of an extra hydroxyl group at the *ortho* or *para* position of the hydroxyl group of phenol. The stability of the phenoxy radical in this case is enhanced by the formation of an intra-molecular hydrogen bond (Andre *et al.*, 2010) (Fig. 2.19).

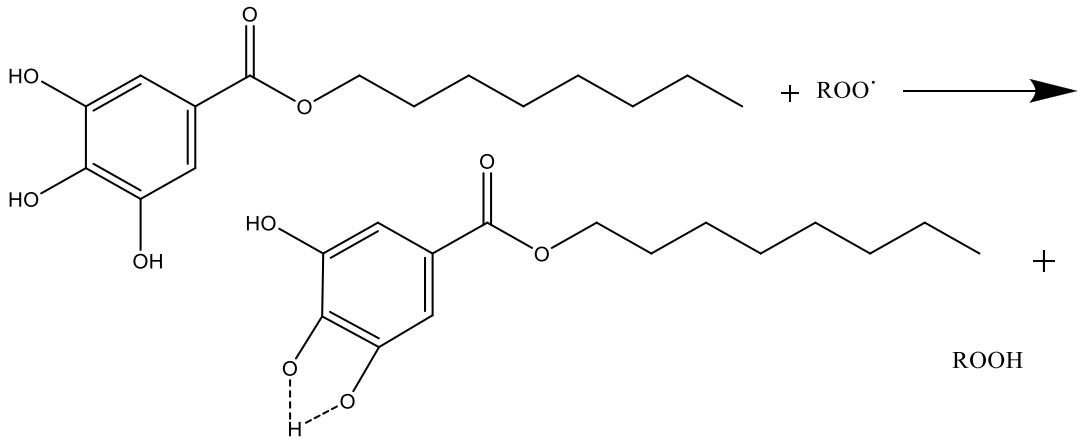


Fig. 2.19. Generation of a phenoxy radical, with the intramolecular hydrogen bond shown

Besides the structure, the antioxidant activity is also affected by other factors such as concentration. The phenolic antioxidants often lose their activity at higher concentrations and rather behave as pro-oxidants participating in the initiation reactions (Becker 1993).

2.7.4.2. Natural Antioxidants

In order to overcome the stability problems of oil and fats, synthetic antioxidants, such as BHA, BHT, tBHQ have been used as food additives (Iqbal *et al.,* 2005) because they are effective and less expensive than natural antioxidants (Suja *et al.,* 2004). But recent reports reveal that these compounds may be implicated in many health risks, including cancer and carcinogenesis (Krings *et al.,* 2000). Therefore, the most powerful synthetic antioxidant (tBHQ) is not allowed for food application in Japan, Canada and Europe. Similarly, BHA and BHT have also been removed from the generally recognized as safe list of compounds (Iqbal *et al.,* 2005, Suja *et al.,* 2004). Due to these safety concerns, there is an increasing trend among food scientists to replace these synthetic antioxidants with natural ones, which, in general, are supposed to be safer.

Lipid peroxidation is one of the major causes of deterioration during storage and food processing. To compensate these deleterious effects, synthetic antioxidant compounds which in excess may contribute to some negative health side effects (Santoso *et al.,* 2004). Therefore, there is a growing interest on the discovery of natural antioxidants, mainly for two reasons: (1) epidemical and clinical evidence suggests that consumption of fruits and vegetables reduce the risk of developing chronic disease, e.g. cancer and coronary heart disorders, and phytochemicals are generally safer than synthetic chemicals (Dastmalchi *et al.,* 2007). For these reasons, many products with antioxidant properties are widely used, particularly from natural sources, in order to minimize oxidative damage to living cells and to prevent oxidative deterioration (Rackova *et al.,* 2007).

Ascorbic acid (vitamin C) is widely known for its antioxidant activity and is therefore used in cosmetics and degenerative disease treatments. Vitamin C is a potent antioxidant for hydrophilic radicals, but poor against lipophilic radicals. Vitamin E is the common name given to a group of lipid-soluble compounds of which tocopherol is the most familiar. It is found in lipoproteins and membranes, and acts to block the chain reaction of lipid peroxidation by scavenging intermediate peroxyl radicals being generated (Haslam 1996) (Fig. 2.20). Carotenoids protect

lipids against peroxidative damage by inactivating singlet oxygen (without degradation) reacting with hydroxyl, superoxide, and peroxyl radicals The antioxidant activity of carotenoids is due to the ability to delocalize unpaired electrons through their structure of conjugated double bonds (Haslam 1996) (Fig. 2.20). Lycopene is also well known for its antioxidant activity (Fig.2.20). Phenolic compounds are commonly found in both edible and non-edible plants, and they have been reported to have multiple biological effects, including antioxidant activity. Crude extracts of fruits, herbs, vegetables, cereals, and other plant materials rich in phenolics are increasingly being used in the food industry because they retard oxidative degradation of lipids and improve the quality and nutritional value of food. (Kähkönen *et al.*, 1999). Phenolic compounds are considered secondary metabolites and are synthesized by plants during normal development, and in response to infections, wounding, UV radiation, and insects. These phytochemical compounds derived from phenylalanine and tyrosines occur ubiquitously in plants and are much diversified (Naczk & Shahidi 2004). Phenolic plant compounds fall into several categories; simple phenolics, phenolic acids (derivatives of cinnamic and benzoic acids), coumarins, flavonoids, stilbenes, tannins, lignans and lignins (Fig. 2.21). Chief among these are the flavonoids which have potent antioxidant activities. Flavonoids are an important class of phenolic compounds, and have potent antioxidant activity. Flavonoids have been shown to be highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals (Bravo 1998) implicated in several diseases (Fig. 2.22). Anthocyanins are probably the largest group of phenolic compounds in the human diet, and their strong antioxidant activities suggest their importance in maintaining health (Velioglu *et al.*, 1998) (Fig. 2.22). Essential oils contain monoterpenes as primary compounds and the effects of many medicinal herbs have been attributed to them. Among various monoterpenes that have antioxidant activity are carvacrol, thymol, terpinene and terpinolene, linalool, and isopulegol.

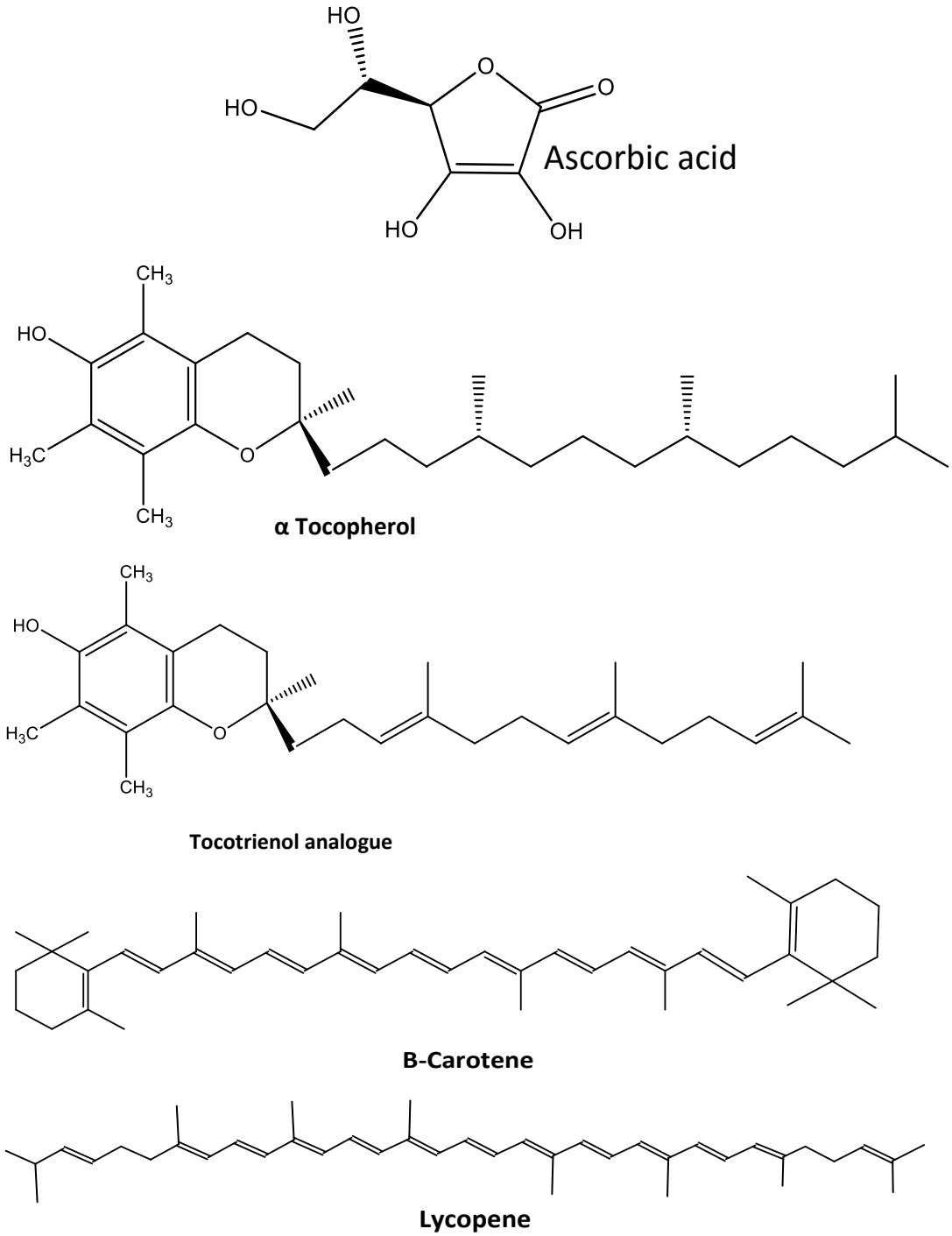


Fig. 2.20. Chemical structures of some natural antioxidant compounds

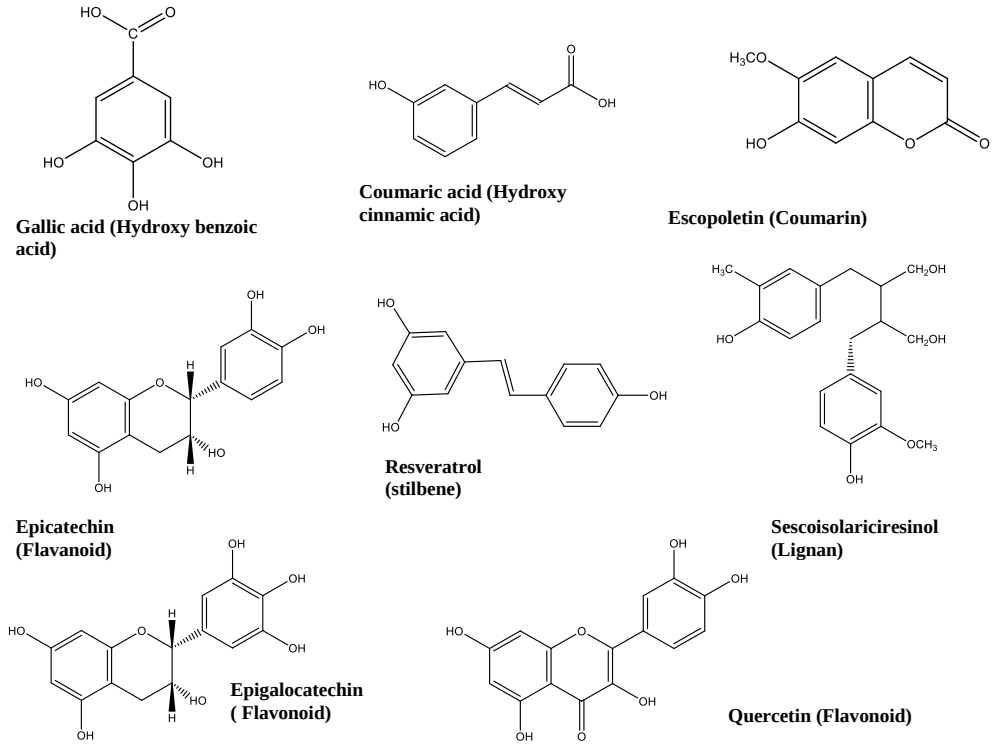


Fig. 2.21. Chemical structures of naturally derived phenolic compounds

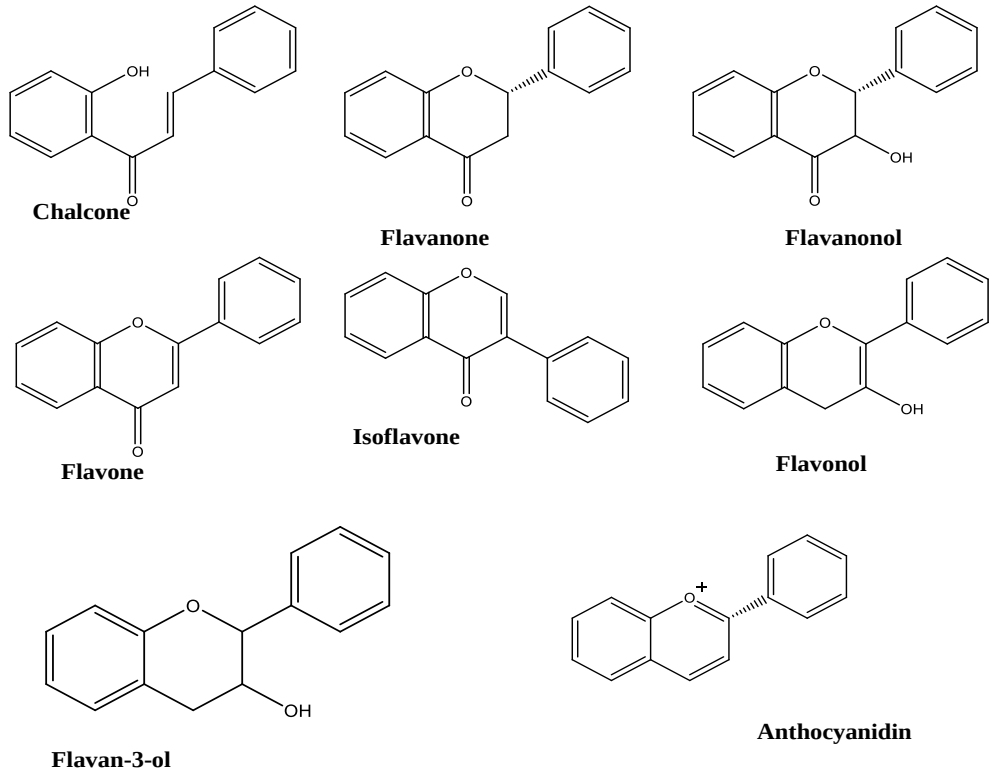


Fig. 2.22. Chemical structures of phenolic antioxidative compounds

2.7.4.2.1. Utilization of Major Natural Antioxidants

The interest in natural antioxidants continues to grow because they are presumed to be safe since they occur in foods and have been used for centuries, and the question of safety of synthetic compounds can thus be avoided (Frankel 1996). Research for a safer and effective natural antioxidant is underway and several natural sources are being examined (Balasundram *et al.,* 2006).

The majority of published work in the area of natural antioxidants has focused on tocopherols (Kalucka *et al.,* 2005). The α -, β -, γ - and δ - tocopherols occur as mixtures in vegetable oils and are the main natural antioxidants in fats (Hraš *et al.,* 2000). α -tocopherol inhibits free radical oxidation by reacting with peroxyl radicals to stop chain propagation, and with the alkoxyl radical to inhibit the decomposition of the hydroperoxides and decrease the formation of aldehydes (Frankel 1996). When tocopherols are mixed with other antioxidants in fish oil, the formulated antioxidant mixtures should be optimized to prevent oxidation. When tocopherols were added with metal chelating agent such as citric acid in fish oil, the stability of fish oil was improved by synergistic effects (Drusch *et al*., 2008). Citric acid can chelate metal ions by forming bonds between the metal and the carboxyl or hydroxyl groups of the citric acid molecule. Citric acid is very effective in retarding the oxidative deterioration of lipids in foods and is commonly added to vegetable oils after deodorisation.

The use of spices and herbs as antioxidants in processed foods is a promising alternative to the use of synthetic antioxidants (Madsen *et al.,* 1998). Numerous reports of anti- oxidative activity of spices have appeared, strongly inspired by an increasing consumer interest in natural food additives. Many spices or their extracts have been assessed for antioxidant activity in a variety of food products and lipid systems (Tsimidou *et al.,* 1995). Most studies have so far concentrated on the anti-oxidative effects of rosemary and their extract (Hraš *et al.,* 2000, Madsen *et al.,* 1998). The compounds responsible for anti-oxidative activity of rosemary are mainly phenolic diterpenes such as carnosic acid, carnosol, rosmanol, epirosmanol, isorosmanol, methyl carnosate and other phenolic acids, such as rosmarinic acid (Hraš *et al.,* 2000, Kalucka *et al.,* 2005). Many rosemary extracts, for use in food systems, are today available on the market. However, the strong and characteristic flavour of rosemary might limit the use of this spice despite the well established and

very high anti-oxidative capacity (Madsen *et al.*, 1998). The antioxidant activity of clove bud extract and its major aroma components, eugenol and eugenyl acetate, was comparable to that of the natural antioxidant, α-tocopherol (vitamin E) (Lee & Shibamoto 2001). Clove bud extract inhibited malonaldehyde formation in cod liver oil. Many works have been carried out on antioxidant actions of tea (Wanasundara & Shahidi 1998, Zandi & Ahmadi 2000, Krings *et al.*, 2000). Green tea leaves contain up to 36% polyphenols, on a dry weight basis and tea extracts are powerful antioxidants owing mainly to the presence of the flavanols epigallocatechin gallate, epicatechin gallate, epigallocatechin and epicatechin. Tea catechins retard oxidation of marine oils with a similar effectiveness as synthetic antioxidants and green tea extracts have the potential for large-scale application as natural antioxidants. Tea catechins are effective scavengers of free radicals and also act as metal chelators. Chlorophyll present in organic extracts from green tea also affects the antioxidant activity of the extracts.

2.8. Antioxidant and Pharmacologically Active Compounds from Marine Ecosystem

The marine world offers an extremely rich resource for important compounds of structurally novel and biologically active metabolites. It also represents a great challenge that requires inputs from various scientific areas to bring the marine chemical diversity up to its therapeutic potential. Marine plants such as seaweeds, sea grass and mangroves also contain high amounts of polyphenols such as phenolic acids, flavonoids, anthocyanidins, lignin, tannins, catechin, epicatechin, epigallocatechin, and gallic acid (Yoshie *et al.*, 2002; Makita *et al.*, 1996). These polyphenolic compounds have shown many health-benefiting bioactivities, such as antioxidant, anticancer, antiviral, anti-inflammatory, and an ability to inhibit human platelet aggregation (Cahyana *et al.*, 1992). Seaweeds are known to contain reactive antioxidant molecules, such as ascorbate and glutathione (GSH) when fresh, as well as secondary metabolites, including carotenoids (α- and β-carotene, fucoxanthin, astaxanthin), mycosporine-like amino acids (mycosporine-glycine) and catechins (e.g., catechin, epigallocatechin), gallate, phlorotannins (e.g., phloroglucinol), eckol and tocopherols (α-, β-, δ- tocopherols). Some studies shown that polyphenols derived from brown algae are potent ferrous ion chelators (Senevirathne *et al.*, 2006) and their metal- chelating ability is dependent on the unique phenolic structure and

the number and location of the hydroxyl groups (Santoso *et al*., 2004). The major role of algal polyphenols therefore appeared to be as potent radical scavengers and primary chain breaking antioxidants (Wang *et al.,* 2009). Reducing power has been suggested as an important indicator of the overall antioxidant capabiity of phenoic antioxidants. It is associated with the presence of reductones, which exhibit their antioxidant activities through the action of breaking the free radical chain by the donation of a H-atom (Gordon 1990). Algal polyphenols may act in a similar way to reductones since they possess the intrinsic ability to donate electrons and react with free radicals, converting them to more stable end product and blocking free radical chain reaction (Duan *et al.,* 2006). Kayalvizhi et al. (2014) reported that the phenolic fraction exhibited higher antioxidant activity in seaweed *Turbinaria ornata* than that in *Padina tetrastromatica.* Tannins, phlorotannins are tannin compounds which have been found only in marine algae. Phlorotannins are formed by the polymerization of phloroglucinol (1, 3, 5-trihydroxybenzene) monomer units and synthesized in the acetate-malonate pathway in marine alga (Targett & Arnold 1998). Phlorotannins purified from several brown algae have been reported to possess strong antioxidant activity which may be associated with their unique molecular skeleton (Ahn *et al.,* 2007). Phlorotannins from brown algae have up to eight interconnected rings. They are therefore more potent free radical scavenger than other polyphenols derived from terrestrial plants, including green tea catechins, which only have three to four rings (Hemat *et al.,* 2007). Some active antioxidant compounds from marine algae were identified as phylopheophylin in *Eisenia bicyclis* (Cahyana *et al.,* 1992) phlorotannins in *Sargassum kjellamanianum* (Yan *et al.,* 1996) and fucoxanthin in *Hijikia fusiformis* (Yan *et al.,* 1998). The biologically active compounds in algal extracts also include poly saccharides comprising galactans, fucoidan, laminarin and alginates (Ferreira *et al*., 2012). The sulphated polysaccharides of Sargassum spp act as a potent free radical scavenger and anticancer agent (Dias *et al.*,2006). According to that novel findings of marine seaweed is a valuable antioxidant source, it consists of high level of antioxidants compounds (Anggadiredja *et al.,* 1997). Fucoxanthin is the main non-provitamin A carotenoid present in brown seaweeds which belongs to the group of xanthophylls and exhibits potent antioxidant activity (Gupta 2011) Algae and consequently their extracts can be the treasure trove of biologically active compounds. Their beneficial properties for humans, animals and plants were

recognized in the past and are appreciated nowadays, in the development of new biotechnological products. Efficacy of biological and chemical compounds contained in alginate brown seaweed, including *Padina sp.* can be used as an antibacterial, anticancer, antioxidant and in overcoming the gland disorder and decreasing high blood pressure (Setha *et al.,* 2013). Eckol is a polyphenolic compound which was isolated from brown algae, *Ecklonia cava* and demostrated cytoprotective effect against oxidative stress (Zhang *et al.,* 2006).

Fig. 2.23. Photographs of seaweed collection site in Southeast coast of India and the collection

Fig. 2.24. Photographs of seaweeds: (A) *Caulerpa Racemosa*; (B) *Chondrococcus hornimani*; (C) *Gracilaria crassa* and (D) *Liagora erecta*.

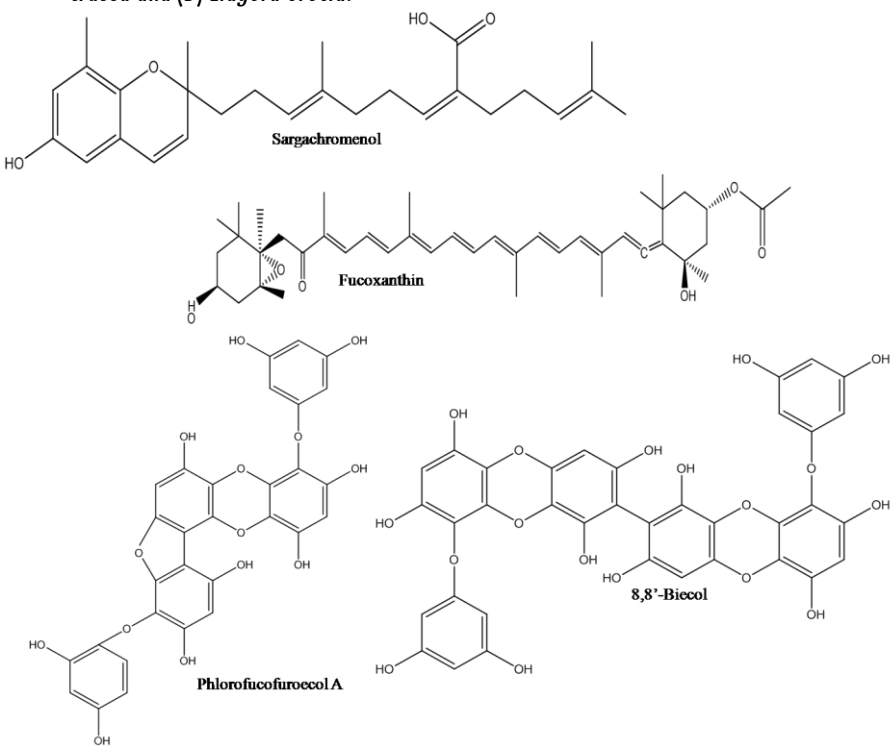


Fig. 2.25. Examples of reported compounds isolated from marine seaweeds

Halophytes are highly salt tolerant plants that can be found in sand dunes or ky coasts, saline depressions or inland deserts, and in marine environments such as coastal salt marshes (Ksouri *et al.*, 2008). In order to withstand the often unfavorable conditions of these locations (e.g., high salinity and UV-irradiation levels), halophytic species have developed several physiological traits that allow them to retain and acquire water, protect cells from the damage caused by the accumulation of reactive oxygen species (ROS), and maintain ion homeostasis (Megdiche *et al.*, 2007). These traits include the biosynthesis of different primary and secondary metabolites, such as vitamins, terpenoids, phenolics, polysaccharides and glycosides, which display several biological activities, including antioxidant, antimicrobial, anti-inflammatory, and antitumoral, and thus they can be crucial for the prevention of a variety of diseases as, for instance, cancer, chronic inflammation and cardiovascular disorders. Several halophytic plants have been used in traditional medicine. Representative examples are the treatment of microbial infections (e.g., *M. edule*, Aizoaceae), to reduce blood pressure (*Salsola kali* L., Chenopodiaceae) or in cancer treatment (*Artemisia scopariae* Waldst. and Kit., Asteraceae), and in many cases *in vitro* studies have confirmed these ethnopharmacological uses (Ksouri *et al.*, 2012).

Fig. 2.26. Photographs of halophytes., (A) *Sesuvium portulacastrum* (Dedicated website: *http://itsgoodthis.com/*) ; (B) Saline desert with predominance of halophytes on the lower section of the Persian Gulf coast (Dedicated website: *http://www.ecosystema.ru/*); (C) Dedicated website: *http://www.greenprophet.com/*

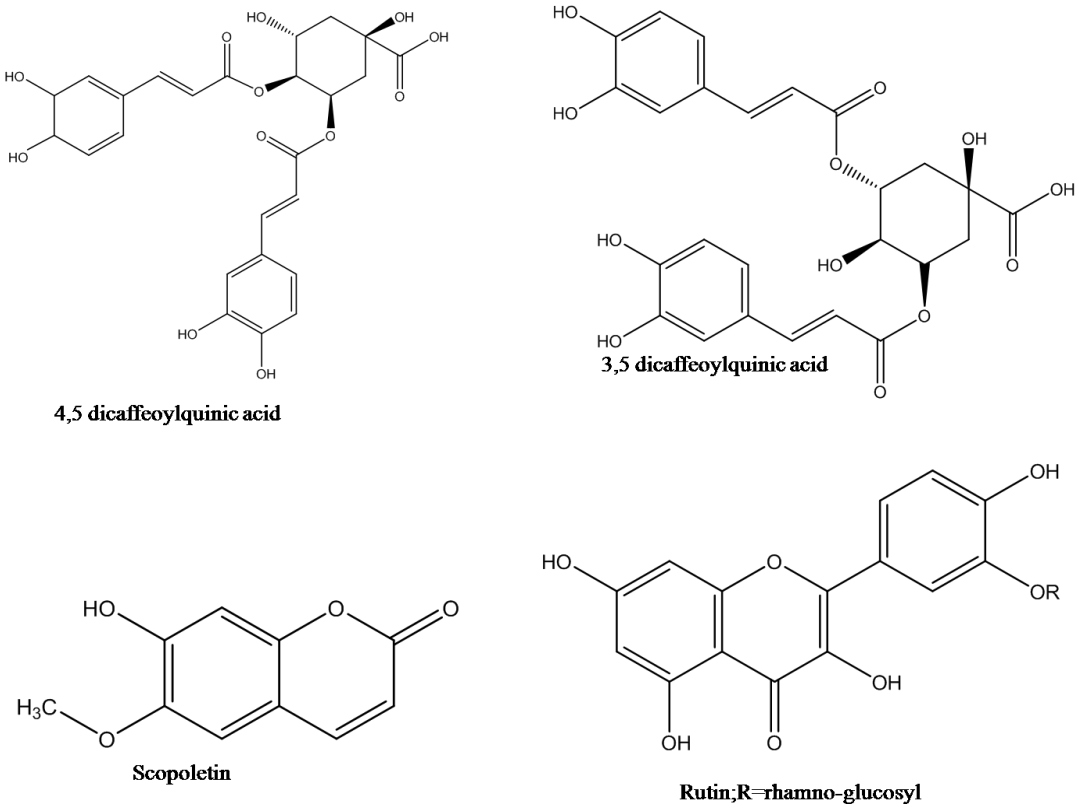


Fig.2.27. Examples of reported compounds isolated from Halophytes

The secondary metabolites derived from number of marine animals possess antibiotic, anti-parasitic, antiviral and anti-cancer activities (Simmons *et al.,* 2005). The Tyrian purple an ancient dye pigment is the first natural product of marine origin reported in literature. Many mollusks mantle cavity produces mucus e.g. Muricid gastropods (rock snail) which defend the developing larvae against microbial infection (Benkendorff *et al.,* 2011). Among marine invertebrates, marine molluscs are the good source of bioactive metabolites. The bioactive compounds extracted from many classes of molluscs exhibit antitumor, antileukemic, antibacterial and antiviral properties (Kamiya *et al.,* 1997; Anand *et al.,* 1997**)**. Marine invertebrates rely solely on innate immune mechanisms that include both humoral and cellular responses. In addition to the venoms, a range of other natural products have been isolated from the Cephalopoda. These include cytotoxic tyrosinase from the ink of the cuttlefish *Sepia officinalis* (Russo *et al.,* 2003), as well as ovarian jelly peptides (Bernay *et al.,* 2006), a sperm-attracting peptide (Zatylny *et al.,* 2002) and novel cardioactive peptide isolated from the brain of the common octopus, *Octopus*

vulgaris (Kanda & Minakata, 2006). The compounds reported from cephalopods in Baker & Murphy (1981) include fairly simple, widely distributed compounds, such as aromatic amino acids, noradrenaline and some benzoquinols.

Fig. 2.28. Photographs of Cephalopods

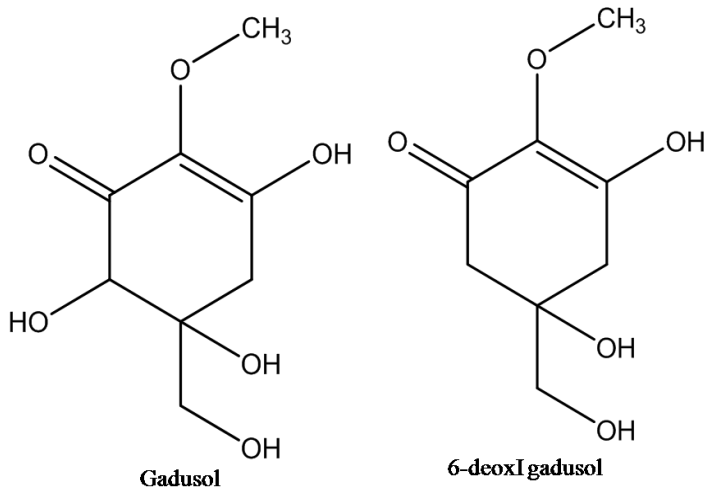


Fig. 2.29. Examples of reported compounds isolated from Cephalopods

Keeping these facts as background information, the present study has attempted to screen locally available low-value fishes for PUFAs, concentrating these essential fatty acids therefrom by chemical and/or enzymatic (lipase-catalyzed) hydrolysis of triglycerides, and to prepare a stabilized aquafeed and/or nutraceutical formulation containing enriched PUFA/s. The present work aims not only to develop an indigenous product, but it will address the hidden facts, which were probably unanswered in the imported versions. Firstly, the imported PUFA concentrates are derived from liver oil extracted from the liver, which filters and detoxifies the blood. Because of this, the liver could contain highly-concentrated levels of toxins,

contaminating any supplements made from it. Because other fish oil supplements are made from other parts of the fish, they are less likely to be contaminated. Another very important fact is that due to the multiple unsaturation in the acyl chain of the PUFAs, they are susceptible to oxidation in the presence of molecular O_2 resulting in loss of quality of the product adversely leading to the formation of undesirable oxidation products, and isomeric conversion of natural all-*cis* PUFAs to their deleterious *trans*-forms, which can be dangerous for immune system. This very important phenomenon is often overlooked by the producers and consumers because we cannot visualize their effect immediately, though these undesirable oxidation products and *trans*-fatty acids will lead to a cascade of immunosystem compromising diseases in long run. It is, therefore, apparent that the PUFA concentrates can be safely used in health food/aquafeed formulations provided their shelf-life and stability is assured. This work further attempted to prepare indigenous product containing stabilized PUFA concentrate with individual or combination of different ingredients with potential antioxidant and other bioactive properties in an attempt to impart stability for health food/aquafeed supplements.

Chapter 3

SCREENING AND SELECTION OF MARINE FISHES FOR POLYUNSATURATED FATTY ACIDS

Contents

Background

The amount and variety of the fatty acids in fish oil varies from species, biological stage, fish diet, fishing location, ocean temperature, nutritional and spawning state, etc (Oterhals 1995, Bandarra *et al.,* 1997, Bandarra *et al.*, 200, Aro et al., 2000). For instance, fish from tropical climate were found to have lower amounts of total lipids compared to fishes from the arctic region. Hence, these factors influence the suitability of the oils for edible and technical applications. On this account, the first target was to analyze the seasonal change in all kinds of fatty acids *viz*., saturated, monounsaturated and polyunsaturated, for the candidate marine fishes.

Four different marine finfish species, ribbon fishes, silver bellies, oil sardines and spiny cheek groupers, have been short listed because they are readily available throughout Southwest and Southeast coasts of India in almost all different seasons. Ribbon fish is a pelagic fish, which occupy an important place among the food fishes of India. The ribbon fishes belong to the family Trichiuridae, and are represented in Indian waters by four species namely, *Trichiurus lepturus, Lepturacanthus savala, Eupleurogrammus intermedius* and *E. muticus.* Among these, *T. lepturus* is most abundant along the Indian coasts of the Arabian Sea and Bay of Bengal (James 1963). Silver bellies are a significant group of finfishes widely distributed in Indo-Pacific waters. The southern coast is the most productive zone for silver belly in India, and occupied about 85% of total landings (Chandrani *et al.,* 2012). Indian oil sardine (*Sardinella longiceps*), a low value fish available abundantly in India, which

shows highly opportunistic feeding behavior are situated close to the bottom of the pelagic food web, and feed directly on available phytoplanktons, mainly diatoms, micro/meso zooplanktons (Garrido *et al.*, 2008). Spinycheek grouper, *Epinephelus diacanthus* is a demersal marine fish species found on the continental shelf of the northern Indian Ocean from the Gulf of Aden to Sri Lanka and India. It is one of the most common groupers on the west coast of the Arabian Sea (8-33% of all groupers landed), but is less common on the east coast fencing the Bay of Bengal, where it contributes less than 5% of grouper landings. The fishes of the sub family Epinephelinae popularly known as groupers or rock cods constitute an important component of demersal fishery resource of India and forms about 2% of total marine landings in India (Banerjee *et al.*, 2006). Groupers have assumed commercial importance in view of their good quality meat and greater consumer demand in local and export markets both in live as well as frozen forms, and is valued as one of the highest quality seafood's in many parts of the world (Chen & Tsai 1994).

Recently, these fishes have found more acceptances because of its extraordinary nutritional qualities particularly PUFAs of the *n*-3 series, such as DHA and EPA. The present study was undertaken to investigate the spatial and seasonal variations in the lipid, fatty acid composition and various fatty acid based nutritional indices of these candidate marine fishes during the pre-monsoon (February - May), monsoon (June - September) and post-monsoon (October - January) seasons along the Southwest (Arabian Sea) and Southeast coasts (Bay of Bengal) of India. This study further evaluated the seasonal variation in the nutritional composition of oil sardines (with respect to proximate composition, amino acid profile, minerals and vitamin content) collected from the Southwest coast of India.

3.1. Materials and Methods

3.1.1. Chemicals, Reagents and Instrumentation

All solvents used were of analytical grade (E-Merck, Darmstadt, Germany) and were redistilled in an all-glass system. Doubly distilled /HPLC grade water was used throughout the work, while all reagents used were of analytical grade (E-Merck or Sigma-Aldrich). Potassium hydroxide, perchloric acid ($HClO_4$), concentrated

nitric acid (HNO_3), ammonium molybdophosphate, 2, 6-dichlorophenol indophenol, boron trifluoride/ methanol (14% BF_3 in MeOH, w/v), triethylamine, sodium phosphate ($NaHPO_4$), phenylisothiocyanate (PITC), sodium acetate trihydrate, standards of fatty acid methyl ester (FAME) (Supelco^{TM} 37 Component FAME Mix, Catalog No. 47885-U) and the vitamin standards (A, D_3, E, K) were procured from E-Merck (Darmstadt, Germany) or Sigma-Aldrich Chemical Co. Inc. (St. Louis, MO). Standards of amino acids were procured from Thermo Scientific (PIERCE amino acid standard H). Mineral standards were purchased from Perkin Elmer (Shelton, USA).

An UV-Visible spectrophotometer (Varian Cary, USA) was used for reading absorbance. The gas-liquid chromatographic (GLC) data of fish fatty acids which converted to FAMEs were recorded on a Perkin-Elmer (USA) AutoSystem XL gas chromatograph (HP 5890 Series II). The amino acid analyses were performed on a high performance liquid chromatography (HPLC, Waters reversed-phase PICO-TAG amino acid analysis system), while the analysis of fat soluble vitamins, A, D_3, E and K were performed on a Shimadzu HPLC (Prominence). The mineral profile was analyzed using an atomic absorption spectrophotometer (AAS, CHEMITO AA 203, Chemito Instruments Pvt. Ltd., India). The solvents were evaporated using a rotary vacuum evaporator (Heidolf, Germany).

3.1.2. Samples and Study Area

The four marine finfishes used in this study were as follows:

1) *Trichiurus lepturus* (Linnaeus, 1758); [Common name - Ribbon fish]
2) *Leiognathus splendens* (Cuvier, 1829); [Common name - Silver bellies]
3) *Sardinella longiceps* (Valenciennes, 1847); [Common name - Indian Oil sardine]
4) *Epinephelus diacanthus* (Valenciennes, 1828); [Common name Spinycheek grouper]

The photographs of the fishes were shown in Fig. 3.1A-D.

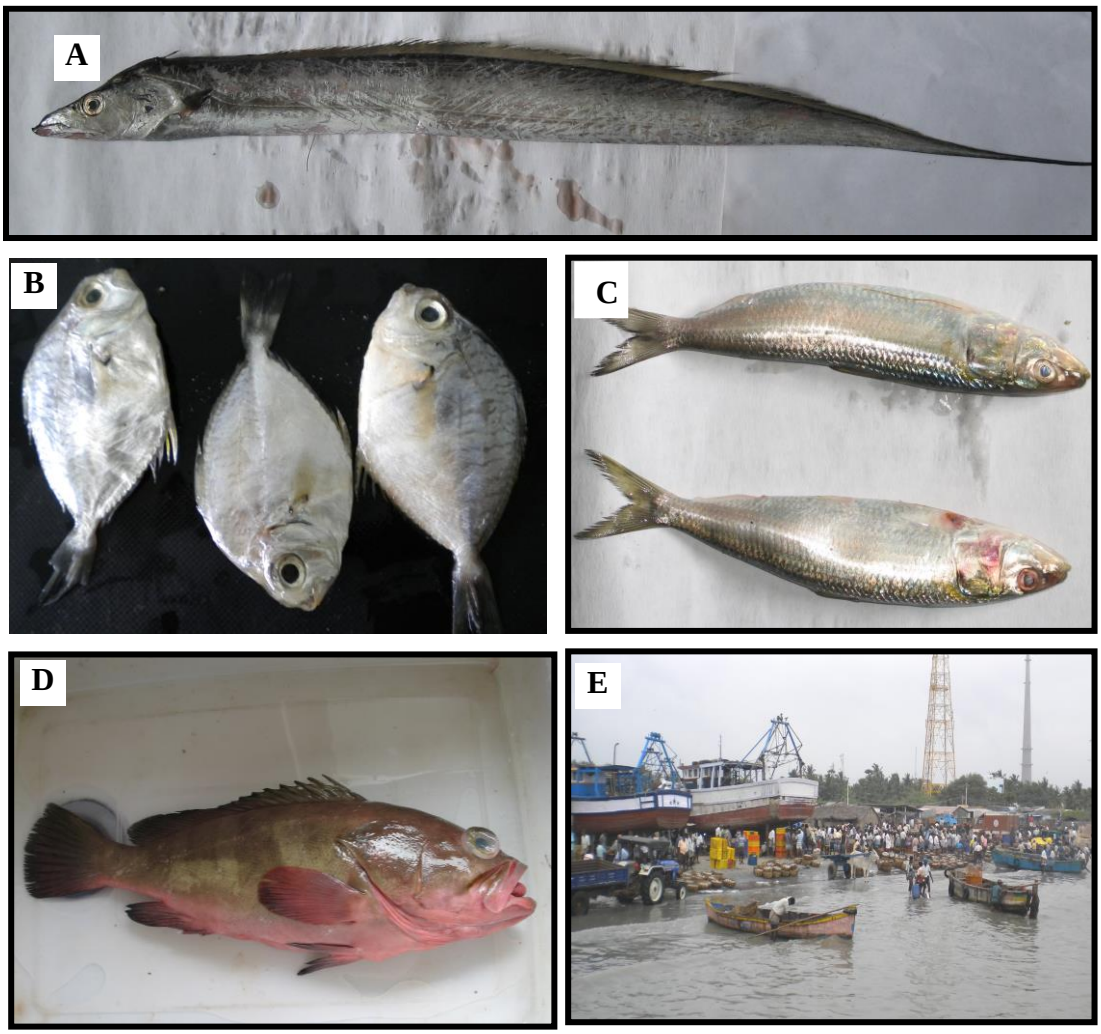


Fig. 3.1. Photographs of candidate marine fishes (**A**) *Trichiurus lepturus*; (**B**) *Leiognathus splendens*; (**C**) *Sardinella longiceps;* (**D**) *Epinephelus diacanthus* and (**E**) a landing centre in South east coast of India

To study the spatial variations in the fatty acid composition of these fishes, they were collected from the Southwest (Fig. 3.2; Mangalore - 12.87°N 74.88°E., Calicut - 11.25°N 75.77°E., and Cochin - 9.97°N 76.28°E) and Southeast (Fig. 3.2; Chennai - 13.08°N 80.27°E., Mandapam - 9.28°N 79.12°E., and Tuticorin - 8.81°N 78.14°E) coasts of India.

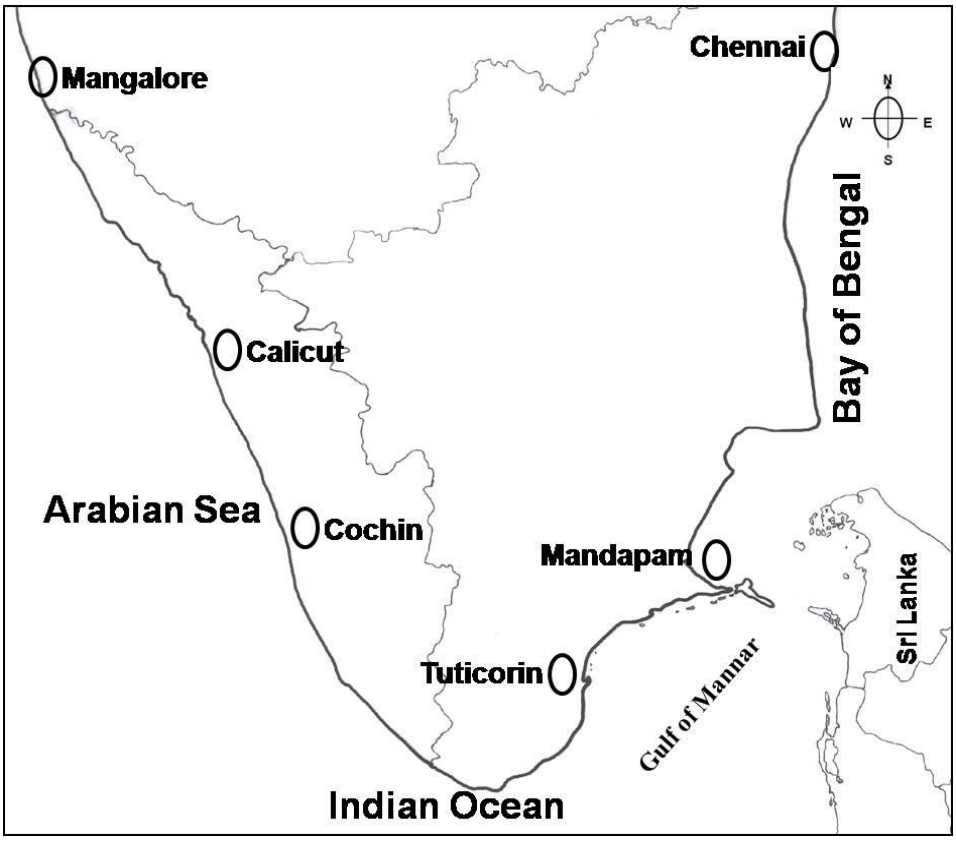


Fig. 3.2. The map showing the sampling sites of the fishes at the Southwest (SW; Mangalore, Calicut and Cochin) and Southeast (SE; Chennai, Mandapam and Tuticorin) coasts of India

The samples were collected from the fishing harbors on the 12-15th day of each month (January - December) of the year, 2010. In order to obtain information on the seasonal variations, the monthly data were grouped as pre-monsoon (February to May), monsoon (June to September) and post- monsoon (October to January). The results of the three centers of each coast were pooled and the mean values were used in the present study. Two pools of fishes per collection site each composed of 15 – 20 specimens of comparable body size were collected within each sampling and were brought to the laboratory in an icebox. After washing in sterile water the internal organs were removed and the meat was cut into slices. Meat was then mixed in a blender for 2 minutes. It is to be noted that although, age and sex differences in nutritional composition could occur, this study regarded the fish as a whole food source, which was representative of the market, and thus totally used by the local population, without any age or sex differences.

3.1.3. Lipid and Fatty acid Profile of the Candidate Marine Fishes

3.1.3.1. Estimation of Total Lipids

100 g of the edible portion of each species were left to extract lipids overnight in the dark in 1 litre of chloroform: methanol (2:1, v/v) and distilled water (200 ml). The lipid extracts were washed according to the Folch procedure (Folch *et al.*, 1957) and the organic layer containing lipids was evaporated *in vacuo*. The lipid content was determined gravimetrically in triplicate and expressed as % (w/w) of the edible portion.

3.1.3.2. Fatty Acid Profiling by Gas-Chromatographic Analysis

The fatty acid compositions of the lipids were performed by GLC according to the established procedure (Chakraborty & Paulraj 2008; Chakraborty & Paulraj 2009). The fatty acid converted to fatty acid methyl esters (FAMEs) were recorded on a Perkin-Elmer (USA) AutoSystem XL gas chromatograph (HP 5890 Series II) connected with a SP 2560 (crossbond 5 % diphenyl- 95 % dimethyl polysiloxane) capillary column (100 m X 0.25 mm i.d., 0.50µm film thickness, Supelco, Bellfonte, PA) using a flame ionization detector (FID) equipped with a split/splitless injector, which was used in the split (1:15) mode. The analyses were accomplished using an oven temperature ramp program: 140 °C for 1 min, rising at 30 °C / min to 250 °C, where it was held for 1.0 min, followed by an increase of 25 °C /min to 285 °C, where it was held for 2.0 min, until all peaks had appeared. The injector and detector were held at 285 and 290 °C, respectively. Nitrogen (ultra high purity > 99.99%) was used as the carrier gas with a pressure of 5.6×10^3 kg/m^2 and flow rate of 25 ml/min. The flow rate of hydrogen (45 ml/min) and air (450 ml/min) were maintained at a pressure of 3.5×10^4 kg/m^2. The injection volume was 0.2 µL. FAMEs were identified by comparing the retention time of the samples and appropriate FAME standards and the relative percentage of the area was obtained by using the subsequent formula: Percent of total fatty acid (% TFA) = (A_{FAME}/A_{TOTAL}) X100; where, A_{FAME} = Area of the methyl esters and A_{TOTAL} = Total area of the chromatogram. Peak areas lower than 0.1% of the total area was not considered.

Three replicate injections were performed and the mean results were expressed as percent of total fatty acids (% TFA) ± standard deviation.

3.1.3.3. Evaluation of Fatty Acid Based Nutritional Indices

The different nutritional indices with respect to fatty acids, such as, *n*-3/*n*-6, *n*-6/*n*-3, DHA/EPA, PUFA/SFA and LA/ALA ratios were calculated in order to allow comparisons with the United Kingdom Department of Health recommendations (HMSO 2001). The indices of atherogenicity (AI) and thrombogenicity (TI) (Ulbricht & Southgate, 1991) have been calculated as: AI = (4 * 14:0 + 16:0 + 18:0) / (MUFA + *n*-3 PUFA + *n*-6 PUFA) ; TI = (14:0 + 16:0 + 18:0) / [(0.5 * MUFA) + (0.5 * *n*-6 PUFA) + (3 * *n*-3 PUFA) + (*n*-3 PUFA/*n*-6 PUFA). The hypocholesterolaemic/ hypercholesterolaemic (HH) ratio were determined as, HH= (18:1*n*-9 + 18:2*n*-6 + 20:4*n*-6 + 18:3*n*-3 + 20:5*n*-3 + 22:5*n*-3 + 22:6*n*-3)/ (14:0 + 16:0) (Santos-Silva *et al.*, 2002).

3.1.4. Nutritional Composition of *Sardinella Longiceps*

3.1.4.1. Proximate Composition

The proximate composition analyses of the edible portion of the fishes were performed as follows: Moisture was determined by oven drying at 105 ºC to constant weight (AOAC 1990). Dried samples were used for determination of crude fat, crude protein and ash contents. All analyses were done in triplicate. The crude protein was determined by the Kjeldahl method (AOAC 1990). Crude fat was extracted from the dried tissues using Bligh and Dyer (1959) method. The fat content was gravimetrically determined. Ash was determined gravimetrically in a muffle furnace by heating at 550 ºC constant weight (AOAC 1990).

3.1.4.2. Determination of True Protein and Amino Acids

The protein contents of the oil sardines were estimated by the established method (Lowry *et al.*, 1951). The absorbance of the protein aliquot was measured at 660 nm in a UV-Visible spectrophotometer within 15 min against the reagent blank. The protein content of the sample was calculated from the standard curve of bovine serum albumin, and expressed as g/100g edible portion. Amino acid content of the

oil sardines was measured using Pico-Tag method as described earlier by Heinrikson and Meredith (1984) using suitable modifications. The sample (0.1 g) was hydrolysed for 24 h at 110 ^{o}C with 6 M HCl in sealed glass tubes filled with nitrogen. The hydrolyzed samples were treated with redrying reagent (MeOH 95%: water: triethylamine, 2:2:1 v/v/v), and thereafter pre-column derivatization of hydrolysable amino acids was performed with phenylisothiocyanate (PITC, or Edman's reagent) to form phenylthiocarbamyl (PTC) amino acids. The reagent was freshly prepared, and the composition of derivatizing reagent (methanol 95%: triethylamine: PITC, 20µL, 7:1: l v/v/v). The derivatized sample (PTC derivative, 20 µL) was diluted with sample diluent (20 µL, 5 mM sodium phosphate $NaHPO_4$ buffer, pH 7.4: acetonitrile 95:5 v/v) before being injected into reversed-phase binary gradient HPLC fitted with a packed column (dimethylocatadecylsilyl- bonded amorphous silica; Nova-Pak C_{18}, 3.9 X 150 mm) maintained at 38±1 °C in a column oven which connected to a Waters 2487 dual absorbance detector (λ_{max} 254 nm). The mobile phase eluents used were eluents A and B, whereas eluent A comprises sodium acetate trihydrate (0.14 M, 940 ml, pH 6.4) containing triethylamine (0.05%), mixed with acetonitrile (60 ml), and eluent B used was acetonitrile : water (60:40, v/v). A gradient elution programme, with increasing eluent B was employed for this purpose. An additional step of 100% eluent B is used to wash the column prior to returning to initial conditions. Standard was run prior to sample injection. Samples (PTC amino acid derivatives) were injected in triplicate, and the output was analyzed using BREEZE software (Waters Corporation, Milford, MA 01757). The quantification of amino acids was carried out by comparing the sample with the standard, and the results were expressed in g/100g edible portion. The total essential amino acids (TEAA), total non-essential amino acids (TNEAA), total amino acids (TAA), total aromatic amino acids (TArAA), total sulphur containing amino acids (TSAA) and the ratios of TEAA to TNEAA, TEAA to TAA, TNEAA to TAA, leucine/isoleucine (Leu/Ileu), arginine/lysine (Arg/Lys), cysteine in total sulphur containing amino acids (Cys/TSAA) were calculated.

3.1.4.3 Estimation of Vitamins

The estimation of fat soluble vitamins (A, D_3, E and K_1) in the edible portions of the oil sardines were carried out using a modified method of Salo-Vaananen *et al*. (2000). The stock solutions of vitamin standards were prepared (1, 10, 25, 50, & 100 ppm) to construct the standard curve by HPLC. All the stock solutions were stored at -20 ^oC except vitamin D_3 (stored at 4^oC). The lipids were extracted using the procedure described in 3.1.3.1, which was hydrolyzed, with KOH/MeOH (0.5N, 2 ml) at 60 ^oC for 30 min to furnish the hydrolyzed mixture, which (2 ml) was thereafter extracted with petroleum ether (12 ml), and washed with distilled water (2 x 8ml) to make it alkali-free. The non-saponifiable matter (8 ml) was concentrated using a rotary evaporator (50 ^oC), reconstituted in MeOH, and filtered through nylon acrodisc syringe filter (0.2 µm) to be injected (20 µL) in HPLC equipped with a C_{18} column (Phenomenex, 250 mm length, 4.6 mm I.D., 5µm) in a column oven (32 ^oC). The run time was 45 min, and the eluents were detected at 265 nm (UV-VIS detector) using the gradient programme as follows: 20% MeOH upto 3 min, which was increased to 100% in next 5 min and held for 37 min. The flow rate was 1 ml/min. The vitamins A, D_3, E, K_1 were expressed as µg/100g edible portion.

The water soluble vitamin, ascorbic acid, was determined based upon the quantitative discoloration of 2, 6-dichlorophenol indophenol titrimetric method as described AOAC (2005). The vitamin C was expressed as µg/100g edible portion.

3.1.4.4. Estimation of Minerals

The estimation of minerals was carried out by atomic absorption spectrophotometer (AAS) following the di-acid ($HNO_3/HClO_4$) digestion method with suitable modifications (Astorga-Espana *et al.*, 2007). In brief, the edible portions of oil sardines (2 g) were placed in digestion tubes, to which concentrated HNO_3 (7 ml) was added, and the content was kept for overnight digestion in a fume hood until no brown fumes appeared. The digestion was continued over the sand bath with $HClO_4$ (6 ml) until the color of the solution became pale yellow to colorless. The solution was thereafter cooled and filtered through Whatman No. 1 filter paper. The filtrate was

diluted with distilled water (50 ml) to be injected in atomic absorption spectrophotometer for determination of minerals. The analyses of Ca, Na, K, Mn, Fe, and Zn were performed by flame atomic absorption spectrophotometry equipped with a hollow cathode lamp containing D_2 lamp background correction system. For Se, continuous flow hydride generator coupled with atomic absorption spectrometer was used. Phosphorus content was analyzed by alkalimetric ammonium molybdophosphate method as described in AOAC official method 964.06 (AOAC 2005). The Macro minerals (Na, K, Ca and P) are represented in mg/100g edible portion and the micro minerals (Fe, Mn, and Zn) are represented in µg/100g edible portion.

3.1.5. Statistical Analysis

Statistical evaluation was carried out with the Statistical Program for Social Sciences 13.0 (SPSS Inc, Chicago, USA, ver. 13.0). Analyses were carried out in triplicate, and the means of all parameters were examined for significance by analysis of variance (ANOVA). Pearson correlation coefficient between biochemical compositions of samples collected was analyzed. The level of significance for all analyses was $p<0.05$.

3.2. Results and Discussion

3.2.1. Lipid Content of the Marine Fishes Collected from Southwest and Southeast Coast of India

The lipid content of the ribbon fishes ranged 2.4 – 3.7 % along SW coast and 1.9 – 6.8 % along SE coast (Table 3.1A & 3.1B). No substantial seasonal variations in the lipid content of ribbon fishes collected from both SW and SE coast of India were observed with regards to pre-monsoon (1.9 - 2.4 %) and monsoon (~3.7 %) seasons. However, significantly higher lipid content (6.8 %) was observed during post-monsoon along the SE coast compared to the SW coast ($p<0.05$). The lipid content of the silver bellies ranged 3.2 – 9.5% along SW coast and 3.6 – 7.1 % along SE coast. In both the coasts, post-monsoon maxima in lipid content were observed (9.5 % in SW & 7.1 % in SE, respectively) compared to other seasons. The lipid content of the oil sardines ranged 6.8 – 8.5% along SW coast and 2.4 – 5.9 % along

SE coast. Interestingly, the lipid content was significantly higher during monsoon (1.1%) and post-monsoon (1.0 %) seasons along SW coast compared to these seasons of SE coast (0.67 & 0.6 %, respectively) ($p<0.05$). In all the three seasons studied, the samples collected from SW coast showed significantly higher lipid content than the SE coast collected samples ($p<0.05$). A monsoon maxima (8.5 %) in lipid content was observed along the SW coast followed by post-monsoon and pre-monsoon seasons (7.7 & 6.8 %, respectively). Njinkoue *et al*. (2002) reported that the lipid content of other *Sardinella* species, such as red muscles of *S. maderensis* showed significantly higher values (21 % fresh weight) ($p<0.05$), while that in red muscles of *S. aurita* showed comparable lipid content (10 % fresh weight) as that of the present study ($p>0.05$). The lipid content of the groupers ranged 0.56 – 1.1 % along SW coast and 0.41 – 0.67 % along the SE coast. A monsoon maxima (1.1 %) in lipid content was observed along the SW coast. In the warm climate, as in pre-monsoon, when water is poor in nutrients and mineral salts, the fish uses the energy depots in the form of lipids and proteins (Keriko *et al.*, 2010) thereby resulting in the reduction of lipid content during pre-monsoon seasons. Among all the four fishes studied, the mean lipid content (mean of three seasons) were found to be the highest for oil sardines collected from SW coast (7.7 %), followed by silver bellies collected from SW and SE coasts (6.7 & 5.3 %, respectively).

3.2.2. Fatty acid Composition of Candidate Marine Fishes Collected from Southwest and Southeast of India

The spatio-seasonal study in the fatty acid compositions of candidate marine fishes are detailed as follows. The fatty acid composition of four fishes collected from the Southwest and Southeast coasts of India are recorded in Table 3.1A & 3.1B, respectively. The indicative chromatograms of fatty acid profile in (A) *Trichiurus lepturus*, (B) *Leiognathus splendens*, (C) *Sardinella longiceps* and (D) *Epinephelus diacanthus* are shown in Fig. 3.3A-D.

3.2.2A. Saturated Fatty Acids

Saturated fatty acids with their high caloric content are primarily used as a source or the storage form of energy. The total saturated fatty acids (∑SFA) in the ribbon fishes ranged from 39.1 – 46.6 % along SW coast and 40.5 – 45.6 % along SE coast (Table 3.1A & 3.1B). Ribbon fishes collected from SW coast observed significantly higher (∑SFA) during pre-monsoon (46.6 %), and along SE coast it was significantly higher (∑SFA) during post-monsoon (45.6 %; $p<0.05$). The ∑SFA in silver bellies ranged from 33.1 – 35.5 % along the SW coast and 36.1 – 39.6 % along SE coast. In both the SW and SE coasts, maximum ∑SFA was observed during monsoon season (35.5 & 39.6 %, respectively). In oil sardines, ∑SFA ranged 31.2 – 37.5 % along the SW coast and 35.6 – 42.1 % along the SE coast. A significantly higher ∑SFA was observed during post-monsoon season (37.5 %) along SW coast and pre-monsoon season (42.1 %) along the SE coast ($p<0.05$). No significant differences were observed for ∑SFA content of groupers along both the SW (42.4 - 46.9 %) and SE (43.0 – 48.8 %) coast irrespective of the seasons ($p>0.05$). A pre-monsoon maxima along SW coast (46.9 %) and post-monsoon maxima along SE coast (48.8 %) were observed with respect to ∑SFA content in groupers. Interestingly, the groupers collected from the SE coast exhibited comparatively higher SFAs than those of the SW coast samples. This could be due to the natural variation in the accumulation of fatty acids and the differences in environmental conditions. The most abundant SFA recorded among all the fishes studied were palmitic acid (16:0) with the mean value highest for groupers (25.9 % along SW coast) and ribbon fishes (28.2 % along SE coast). Similar results for other marine fish species have also been reported earlier (Celik *et al.*, 2005). 16:0 is considered to be a source of potential metabolic energy in fish. The mean ∑SFA was found to be the highest for ribbon fishes collected from SW coast (45.7 %), followed by groupers collected from both the SW and SE coast (45.3 and 45.1 %, respectively).

3.2.2B. Monounsaturated Fatty Acids

The total monounsaturated fatty acids (∑MUFA) in the ribbon fishes ranged from 26.4 – 32.5 % along SW coast and 18 – 30.8 % along SE coast. Both the coasts recorded pre-monsoon maxima in ∑MUFA for ribbon fishes, with 32.5 % along SW

and 30.8 % along SE coast. The ∑MUFA in silver bellies ranged from 28.8 – 31.2 % along the SW coast and 23.2 – 25.5 % along SE coast. A post-monsoon maxima along SW coast (31.2 %) and pre-monsoon maxima along SE coast (25.5 %) in ∑MUFA content were observed for silver bellies. However, in oil sardines, during pre-monsoon season, maximum ∑MUFA content was observed along both SW and SE coasts (29.3 & 22.5 %, respectively). ∑MUFA ranged 26.3 – 29.3 % along SW coast and 18.8 – 22.5 % along SE coast for oil sardines. The ∑MUFA content of groupers ranged 25.2 – 27.1 % along SW and 25.3 – 27.7 % along SE coast with a post-monsoon maxima along both SW and SE coasts (Table 3.1A & 3.1B). The most abundant MUFA among the studied marine fishes from both the coasts and all the seasons, except the oil sardines collected from SE coast during pre/post-monsoon seasons, were 18:1*n*-9 (oleic acid). This finding was in agreement with prior findings that the major MUFA detected in marine lipids usually contains 18 carbon atoms (Zlatanos & Laskaridis 2007; Abbas *et al.*, 2009). A mean 18:1*n*-9 maxima was observed in silver bellies collected from SW coast (19.7 %) and groupers collected from SE coast (15.3 %). 16:1*n*-7 was the predominant MUFA in oil sardines collected from SE coast during pre/post-monsoon seasons (9.7 & 10.2 %, respectively). It can be concluded that the low MUFA levels on the SE coast are related to the high concentrations of 16:1*n*-7 in their tissues. The mean ∑MUFA was found to be the highest for ribbon fishes and silver bellies collected from SW coast (30.1 %), followed by oil sardines collected from the SW coast (27.4 %) and groupers collected from the SE coast (26.5 %).

3.2.2C. Polyunsaturated Fatty Acids

The total polyunsaturated fatty acids (∑PUFA) in the ribbon fishes ranged from 19.5 - 28.4 % along SW coast and 17.6 - 36.7 % along SE coast. Both the coasts recorded significantly higher ∑PUFA during monsoon season for ribbon fishes, with 28.4% along SW and 36.7% along SE coast ($p<0.05$). Similarly, a monsoon maxima was observed in ∑PUFA content for silver bellies collected from

both SW and SE coast of India (27.1 & 25.9 %, respectively). The ∑PUFA in silver bellies ranged from 21.7 - 27.1 % along SW coast and 24 - 25.9 % along SE coast. The ∑PUFA ranged 23.5 - 31.5 % along SW coast and 20.6 - 26.8 % along SE coast for oil sardines. A post-monsoon maxima along SW coast (31.5 %) and monsoon maxima along SE coast (26.8 %) in ∑PUFA content were observed for oil sardines. A study by Shirai *et al*. (2002) using *Sardinops melanostictus* caught in Japan showed SFA fractions of 34.3 - 41.4 %, MUFA fractions of 15.1 - 22.4 %, and PUFA fractions of 37.3 - 49.4 %. These levels were in agreement with the present study. However, Shirai *et al*. (2002) reported a higher DHA concentration compared to EPA. Sardines caught in California (*Sardinops sagax* caeruleus) showed a similar ratio of EPA to DHA as in the present study. However, the California sardines had lower overall levels of EPA and DHA (Gamez-Meza *et al.*, 1999). Sardines caught near Astoria showed higher amounts of *n*-3 PUFAs compared to other species such as *Sardina pilchardus* caught in Spain (Beltran & Moral 1991) and *Sardinops melanostictus* caught in Japan (Shirai *et al.*, 2002). During post-monsoon the sea water temperature falls to 25 - 26 °C as compared to 30 – 31 °C and hence fatty acid concentration in fishes like sardines increase to sustain the lowered temperature. This has also been observed in several prior studies on Sardines elsewhere (Gamez-Mesa *et al.*, 1999; Bandarra *et al.*, 1997; Shirai *et al.*, 2002) including one on *S. longiceps* in the tropic coasts (Gopakumar 1965). The ∑PUFA content of groupers ranged 17.6 - 25.1 % along SW and 14.2 - 16.2 % along SE coast with post-monsoon maxima (25.1 % in SW and 16.2 % in SE) in ∑PUFA content (Table 3.1A & 3.1B). These results are in accordance with the earlier studies that PUFA content in fish varies inversely with water temperature (Mateos *et al.*, 2010; Gamez-Mesa *et al.*, 1999; Shirai *et al.*, 2002). The increasing degree of unsaturation of fish lipids with low temperatures is a known strategy used by aquatic organisms to adapt the fluidity and permeability of their cell membranes to the temperature fluctuations of the water (Caramujo *et al.*, 2008). Another reason for the fluctuation in ∑PUFA content could be due to the dominance of juveniles and immatures in the catch during particular

period, as these individuals are heavy feeders and accumulate large quantities of fat in their body tissues.

Among *n*-3 fatty acids, EPA content observed to be maximum for oil sardines (12.8 - 17.1 % in SW & 9.5 – 11.2 % in SE) and silver bellies (9.2 - 9.8 % in SW & 10.5 – 11.5 % in SE), while higher DHA content was observed in ribbon fishes (12.2 – 15.5 % in SW & 5.1 – 25.8 % in SE) and groupers (7.8 – 11.0 % in SW & 6.3 – 7.5 % in SE) (Table 3.1A & 3.1B). The predominance of EPA in oil sardine apparently resulted from its diet, as it appeared to be accumulated from the phytoplanktons, largely unchanged, in the lipids of marine fish due to their reduced capacity of chain elongation and desaturation (Alasalvara *et al.*, 2002). Similarly, the dominance of DHA over EPA concentration in ribbon fishes and groupers could be a result of the presence of additional amount of zooplanktons in the diet of *T. lepturus* ($p<0.05$). However, total EPA +DHA was found to be highest in oil sardines collected from the SW coast (18 - 23.4 %). The mean total EPA +DHA of the fishes collected from SW coast were found to be in the order: oil sardines (20.9 %) > ribbon fishes (18.5 %) > silver bellies (15.9%) > groupers (13.3 %). Similarly, the fishes collected from the SE coast recorded mean total EPA +DHA in the order: ribbon fishes (20.7 %) > oil sardines (18 %) > silver bellies (17.7 %) > groupers (9.9 %). Seasonally, all the four studied fishes collected during monsoon/post-monsoon seasons showed significantly higher total EPA + DHA along the SW coast compared to pre-monsoon season. The total *n*-3 PUFAs were recorded to be substantially higher in all the four studied fishes collected during monsoon/post-monsoon seasons along the SW coast compared to pre-monsoon season. The total *n*-3 PUFAs ranged 16.1 – 22.6 % in ribbon fishes, 18.4 -19.5 % in silver bellies, 19.5 - 25.9 % in oil sardines and 13 -19.7 % in groupers along SW coast. Along SE coast, total *n*-3 PUFAs ranged 13.3 – 33.3 % in ribbon fishes, 19.3 – 20.4 % in silver bellies, 16.7 – 21.3% in oil sardines and 10.4 – 11.6% in groupers. Njinkoue *et al*. (2002) reported that the *n*-3 PUFA contents of *S. maderensis* and *S. aurita* varied between 16 - 32%, which was comparable to the

present study. Another point to be noted that total *n*-3 PUFAs was found to be higher than those of *n*-6 PUFAs over the studied periods. The results on the seasonal variations of EPA and DHA are in good agreement with previous reports carried out on seasonal variations of fatty acid compositions of Japanese sardine, *Sardinops melanostictus* (Shirai *et al.*, 2002). A reduced DHA content with the proportional increase of EPA was recorded in the present study, which may be attributed to the dietary variation (Table 3.1). The result that EPA and DHA values are complementary among the species is particularly interesting from the perspective of a pharmaceutical industry as the result has direct implication on the quality of the fatty acid extracts of oil sardine.

Among *n*-6 fatty acids, 18:2*n*-6 (LA) was observed to be maximum during pre-monsoon and post-monsoon seasons for ribbon fishes, silver bellies and oil sardines collected from SW coast. However, during monsoon, arachidonic acid (20:4*n*-6, AA) was observed to be maximum for all these species collected from SW coast. Groupers collected from SW coast recorded 18:2*n*-6 maxima during pre-monsoon and monsoon, while 18:3*n*-6 was found to be highest during post-monsoon. In SE coast, except silver bellies all other fishes recorded 18:2*n*-6 as the major *n*-6 fatty acids during all the seasons. In contrast, silver bellies collected from SE coast possessed 18:3*n*-6 maxima during all the seasons. Along SW coast, total *n*-6 PUFAs was found to be maximum during monsoon for ribbon fishes (5.3 %), silver bellies (6.8 %) and oil sardines (5.6 %), while, groupers exhibited post-monsoon maxima in *n*-6 PUFA (4.7 %). However, along SE coast, ribbon fishes, oil sardines and groupers recorded maximum ∑*n*-6 PUFA during post-monsoon season (3.6, 5.2 & 4.2 %, respectively) and silver bellies recorded maximum ∑*n*-6 PUFA during monsoon season (4.9 %).

Table 3.1A Lipid (%) and fatty acid composition (% total fatty acids) of the candidate marine fishes collected from the Southwest coast of India over three different seasons (pre-monsoon, monsoon and post-monsoon)

	Trichiurus lepturus			*Leiognathus splendens*			*Sardinella longiceps*			*Epinephelus diacanthus*		
	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon
Lipid(%)	2.38±0.24^{a}	3.69±0.37^{a}	2.93±0.29^{a}	7.41±0.74^{b}	3.2±0.32^{a}	9.53±0.95^{b}	6.8±0.68^{ab}	8.5±0.85^{b}	7.7±0.77^{b}	0.56±0.06^{c}	1.14±0.11^{ac}	1.02±0.1^{ac}
Saturated												
12:0	0.18±0.02^{a}	0.15±0.02^{a}	0.11±0.02^{a}	0.21±0.02^{a}	0.23±0.02^{a}	0.13±0.01^{a}	0.12±0.01^{a}	0.62±0.06^{b}	0.12±0.01^{a}	0.18±0.02^{a}	0.22±0.02^{a}	0.21±0.02^{a}
14:0	6.02±0.6^{a}	4.48±0.46^{ac}	6.13±0.61^{a}	3.89±0.39^{bc}	4.5±0.44^{ab}	3.32±0.35^{b}	2.35±0.24^{b}	8.6±0.87^{a}	9.02±0.9^{a}	4.27±0.43^{ac}	4.06±0.41^{ac}	4.25±0.44^{ac}
15:0	0.79±0.07^{ab}	0.58±0.06^{b}	1.11±0.11^{a}	1.29±0.14^{a}	1.34±0.13^{a}	0.94±0.09^{a}	0.54±0.05^{b}	0.9±0.09^{a}	0.76±0.08^{a}	1.22±0.12^{a}	1.43±0.14^{a}	1.24±0.12^{a}
16:0	25.9±0.59^{a}	24.2±0.42^{a}	23.1±0.3^{a}	16.6±0.66^{b}	17.3±0.73^{b}	18.6±0.88^{b}	21.9±0.19^{a}	14.2±0.42^{b}	21.1±0.09^{a}	25.6±0.51^{a}	27.1±0.69^{a}	25.1±0.53^{a}
17:0	0.8±0.09^{a}	0.9±0.09^{a}	1.16±0.12^{ab}	0.58±0.06^{a}	0.86±0.11^{a}	0.83±0.07^{a}	0.37±0.04^{a}	0.39±0.04^{a}	0.66±0.07^{a}	1.81±0.18^{b}	1.85±0.19^{b}	0.45±0.05^{a}
18:0	11.6±1.15^{a}	7.77±0.77^{bc}	8.68±0.87^{b}	8.8±0.88^{b}	10±1^{ab}	9.02±0.9^{ab}	5.8±0.55^{c}	5.07±0.52^{c}	3.56±0.36^{c}	12±1.2^{a}	10.7±1.05^{ab}	9.54±0.95^{ab}
20:0	0.75±0.08^{a}	0.53±0.05^{a}	0.77±0.07^{a}	0.78±0.09^{a}	0.72±0.07^{a}	0.79±0.08^{a}	0.42±0.04^{a}	0.45±0.05^{a}	1.8±0.17^{a}	0.85±0.08^{a}	0.63±0.06^{a}	0.86±0.09^{a}
22:0	0.46±0.04^{ab}	0.33±0.02^{ab}	0.12±0.01^{a}	0.69±0.07^{b}	0.37±0.04^{ab}	0.87±0.09^{b}	0.25±0.03^{ab}	0.11±0.01^{a}	0.24±0.02^{a}	0.57±0.06^{ab}	0.48±0.05^{ab}	0.68±0.07^{ab}
24:0	0.06±0.01^{a}	0.12±0.01^{ab}	0.06±0.01^{a}	0.29±0.02^{ab}	0.22±0.03^{ab}	0.35±0.05^{b}	2.2±0.22^{c}	0.9±0.1^{ab}	0.25±0.03^{ab}	0.38±0.04^{ab}	0.15±0.02^{ab}	0.12±0.01^{ab}
∑SFA	46.56±1.65^{a}	39.06±3.9^{a}	41.24±2.12^{a}	33.13±1.33^{b}	35.54±1.57^{ab}	34.85±1.52^{b}	33.95±2.37^{b}	31.24±3.16^{b}	37.51±0.73^{a}	46.88±1.64^{a}	46.62±0.63^{a}	42.45±2.28^{a}
Monounsaturated												
14:1*n*-7	0.07±0.01^{a}	0.22±0.02^{a}	0.27±0.03^{a}	0.21±0.02^{a}	0.1±0.01^{a}	0.29±0.03^{a}	0.09±0.01^{a}	0.1±0.01^{a}	0.07±0.01^{a}	0.55±0.06^{a}	0.34±0.03^{a}	0.32±0.03^{a}
15:1*n*-7	0.14±0.01^{a}	0.2±0.02^{a}	0.16±0.02^{a}	0.11±0.01^{a}	0.09±0.01^{a}	0.16±0.02^{a}	0.07±0.01^{a}	ND	0.18±0.02^{a}	0.27±0.03^{a}	0.09±0.01^{a}	0.21±0.02^{a}
16:1*n*-7	5.65±0.57^{a}	7.77±0.78^{ab}	6.93±0.69^{a}	6.59±0.66^{a}	6.45±0.66^{a}	6.55±0.66^{a}	10.6±1.06^{b}	5.9±0.59^{a}	9.94±0.99^{b}	6±0.6^{a}	6.07±0.61^{a}	7.24±0.72^{ab}
18:1*n*-7	0.28±0.03^{a}	0.15±0.02^{a}	0.19±0.02^{a}	0.42±0.04^{a}	0.41±0.04^{a}	0.39±0.04^{a}	0.1±0.01^{a}	ND	0.15±0.02^{a}	0.42±0.04^{a}	0.4±0.04^{a}	0.21±0.01^{a}
18:1*n*-9	23.2±1.33^{a}	20.1±1.02^{a}	15.7±0.57^{b}	18.1±0.82^{ab}	17.2±1.72^{ab}	19.1±1.92^{a}	14.8±1.45^{b}	19.7±1.97^{a}	13.8±1.37^{b}	14.7±1.45^{b}	13.7±1.37^{b}	16.2±1.62^{b}
20:1*n*-9	0.52±0.05^{a}	0.07±0.01^{b}	0.95±0.1^{a}	0.83±0.08^{a}	1.05±0.12^{a}	0.37±0.04^{a}	0.42±0.04^{a}	0.11±0.01^{a}	0.73±0.07^{a}	1.08±0.11^{a}	0.89±0.09^{a}	0.25±0.03^{a}
22:1*n*-9	2.04±0.2^{a}	1.59±0.16^{a}	2.13±0.2^{a}	3.73±0.38^{a}	3.1±0.31^{a}	3.74±0.37^{a}	3.02±0.3^{a}	ND	1.54±0.15^{a}	2.77±0.28^{a}	3.55±0.36^{a}	2.32±0.23^{a}
24:1 *n*-9	0.57±0.06^{a}	1.19±0.12^{a}	0.12±0.01^{a}	0.45±0.05^{a}	0.38±0.04^{a}	0.57±0.06^{a}	0.17±0.02^{a}	0.5±0.05^{a}	0.29±0.03^{a}	0.27±0.03^{a}	0.19±0.02^{a}	0.35±0.04^{a}
∑MUFA	32.47±0.26^{a}	31.29±3.15^{a}	26.45±2.64^{ab}	30.44±3.06^{a}	28.78±2.91^{a}	31.17±3.14^{a}	29.27±2.9^{a}	26.31±2.63^{ab}	26.7±1.66^{ab}	26.06±2.6^{ab}	25.23±2.53^{b}	27.1±2.7^{ab}
Polyunsaturated												
16:2*n*-4	0.44±0.04^{a}	0.16±0.02^{a}	0.37±0.04^{a}	0.46±0.05^{a}	0.61±0.06^{a}	0.2±0.02^{a}	0.13±0.01^{a}	ND	0.35±0.04^{a}	0.65±0.07^{a}	0.21±0.02^{a}	0.54±0.05^{a}
16:3*n*-4	0.22±0.02^{a}	0.16±0.02^{a}	0.23±0.02^{a}	0.24±0.02^{a}	0.24±0.02^{a}	0.25±0.03^{a}	0.35±0.04^{a}	ND	0.35±0.04^{a}	0.14±0.01^{a}	0.12±0.01^{a}	0.19±0.02^{a}
18:2*n*-6 LA	0.96±0.1^{a}	1.46±0.15^{a}	1.5±0.15^{a}	1.38±0.14^{a}	1.68±0.17^{a}	1.24±0.12^{a}	1.37±0.14^{a}	1.24±0.12^{a}	2.04±0.2^{a}	1.28±0.13^{a}	1.25±0.13^{a}	1.14±0.11^{a}
18:3*n*-6	0.87±0.09^{a}	1.01±0.1^{a}	0.56±0.06^{a}	1.18±0.12^{a}	1.84±0.18^{a}	1.02±0.1^{a}	0.73±0.07^{a}	1.92±0.19^{a}	0.66±0.07^{a}	0.71±0.07^{a}	1.24±0.12^{a}	2.02±0.2^{a}
18:3*n*-3 ALA	0.16±0.02^{a}	1.29±0.13^{a}	0.18±0.02^{a}	0.73±0.07^{a}	1.13±0.12^{a}	0.54±0.05^{a}	0.63±0.06^{a}	1.87±0.18^{a}	0.85±0.09^{a}	0.34±0.03^{a}	0.64±0.06^{a}	1.25±0.14^{a}
18:4*n*-3	ND	0.19±0.02^{a}	ND	ND	0.26±0.03^{a}	0.13±0.01^{a}	0.3±0.03^{a}	0.16±0.02^{a}	ND	0.03±0^{a}	0.12±0.01^{a}	0.28±0.03^{a}
20:2*n*-6	0.45±0.05^{a}	0.43±0.04^{a}	0.76±0.08^{a}	0.83±0.08^{a}	1.02±0.1^{a}	0.79±0.08^{a}	0.86±0.08^{a}	0.06±0.01^{a}	1.63±0.16^{a}	1.25±0.13^{a}	1.21±0.13^{a}	0.85±0.09^{a}
20:3*n*-6	0.12±0.01^{a}	0.36±0.04^{a}	0.24±0.02^{a}	0.27±0.03^{a}	0.39±0.04^{a}	0.26±0.03^{a}	0.11±0.01^{a}	0.33±0.03^{a}	0.1±0.01^{a}	0.2±0.02^{a}	0.21±0.02^{a}	0.27±0.03^{a}
20:4*n*-6	0.29±0.03^{a}	2.07±0.21^{b}	0.68±0.07^{a}	0.88±0.09^{a}	1.88±0.2^{ab}	0.39±0.04^{a}	0.45±0.05^{a}	2.09±0.21^{b}	0.52±0.05^{a}	0.36±0.04^{a}	0.35±0.04^{a}	0.38±0.04^{a}
20:5*n*-3 EPA	2.49±0.25^{a}	6.07±0.61^{b}	6.46±0.65^{b}	9.36±0.94^{bc}	9.26±0.93^{bc}	9.83±0.97^{bc}	12.8±1.27^{c}	17.1±1.7d	15.2±1.52^{cd}	3.81±0.37^{a}	4.45±0.45^{ab}	3.65±0.38^{ab}
22:5*n*-3	1.26±0.13^{a}	2.02±0.1^{a}	0.99±0.1^{a}	0.95±0.1^{a}	1.4±0.14^{a}	1.67±0.17^{a}	0.55±0.06^{a}	1.92±0.19^{a}	1.58±0.15^{a}	0.98±0.1^{a}	1.12±0.11^{a}	3.56±0.36^{a}
22:6*n*-3 DHA	12.2±1.22^{a}	13.2±1.32^{a}	15±1.4^{a}	5.45±0.54^{b}	7.43±0.76^{bc}	6.24±0.62^{bc}	5.26±0.53^{bc}	4.22±0.42^{b}	8.25±0.83^{c}	7.85±0.79^{bc}	9.12±0.91^{bc}	11±1.1^{ac}
∑PUFA	19.46±1.96^{a}	28.42±1.76^{b}	26.97±0.61^{bc}	21.73±0.18^{a}	27.14±0.75^{b}	22.56±1.24^{ab}	23.54±2.35^{ac}	30.91±3.07^{b}	31.53±1.16^{b}	17.6±1.76^{a}	20.04±2.01^{ab}	25.13±1.55^{bc}

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. Mean values with different superscripts (a, b etc) in the same row indicates a statistical difference ($p<0.05$). ND: not detected. Minor fatty acids are not reported (< 0.1%).

Table 3.1B Lipid (%) and fatty acid composition (% total fatty acids) of the candidate marine fishes collected from the Southeast coast of India over three different seasons (pre-monsoon, monsoon and post-monsoon)

	Trichiurus lepturus			*Leiognathus splendens*			*Sardinella longiceps*			*Epinephelus diacanthus*		
	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon
Lipid(%)	1.88±0.19^{ac}	3.72±0.37^{a}	6.75±0.68^{b}	5.22±0.52^{ab}	3.62±0.36^{a}	7.09±0.71^{b}	2.4±0.24^{a}	5.9±0.59^{ab}	4.6±0.46^{ab}	0.41±0.04^{c}	0.67±0.07^{c}	0.6±0.06^{c}
Saturated												
12:0	0.1±0.01^{a}	0.16±0.02^{ab}	0.09±0.01^{a}	0.32±0.03^{ab}	0.24±0.02^{ab}	0.13±0.01^{ab}	0.16±0.02^{ab}	0.13±0.01^{ab}	0.09±0.01^{a}	1.62±0.16^{b}	0.2±0.02^{ab}	ND
14:0	1.2±0.12^{a}	2.45±0.25^{a}	8.7±0.87^{b}	4.48±0.45^{ab}	4.91±0.49^{ab}	4.08±0.41^{ab}	8.56±0.86^{b}	7.94±0.79^{b}	8.25±0.83^{b}	4.6±0.45^{ab}	4.01±0.4^{ab}	4.21±0.42^{ab}
15:0	0.65±0.07^{a}	1.45±0.15^{a}	0.5±0.05^{a}	2.22±0.22^{a}	1.6±0.16^{a}	1.59±0.16^{a}	0.85±0.09^{a}	1.07±0.11^{a}	0.65±0.07^{a}	1.05±0.11^{a}	1.37±0.14^{a}	1.23±0.12^{a}
16:0	30.1±3.01^{a}	26.2±2.62^{a}	28.2±2.82^{a}	18.4±1.84^{b}	18.4±1.85^{b}	18.2±1.82^{b}	23.5±2.35^{a}	20.2±2.01^{ab}	18.2±1.82^{b}	28.9±2.89^{a}	25.1±2.51^{a}	28.6±2.86^{a}
17:0	0.58±0.05^{a}	0.83±0.08^{a}	0.32±0.03^{a}	2.03±0.2^{a}	1.77±0.18^{a}	0.48±0.05^{a}	0.79±0.08^{a}	0.68±0.07^{a}	0.52±0.05^{a}	1.43±0.14^{a}	1.24±0.12^{a}	0.68±0.07^{a}
18:0	7.18±0.72^{ab}	8.35±0.84^{ab}	7.07±0.71^{ab}	7.9±0.79^{ab}	11.3±1.13^{b}	10.2±1.02^{ab}	6.45±0.65^{a}	6.4±0.64^{a}	6.5±0.65^{a}	8.56±0.86^{ab}	10.6±1.06^{ab}	12.3±1.22^{b}
20:0	0.27±0.03^{a}	0.41±0.04^{a}	0.3±0.03^{a}	0.59±0.06^{a}	0.83±0.08^{a}	0.8±0.08^{a}	0.75±0.08^{a}	0.47±0.05^{a}	1.12±0.1^{a}	0.68±0.07^{a}	0.53±0.05^{a}	0.72±0.07^{a}
22:0	0.35±0.04^{a}	0.21±0.02^{a}	0.28±0.03^{a}	0.53±0.05^{a}	0.4±0.04^{a}	0.39±0.04^{a}	0.34±0.03^{a}	0.27±0.03^{a}	0.23±0.02^{a}	0.3±0.03^{a}	0.26±0.03^{a}	0.49±0.05^{a}
24:0	0.03±0^{a}	1.06±0.11^{a}	0.09±0.01^{a}	0.28±0.03^{a}	0.15±0.02^{a}	0.21±0.02^{a}	0.69±0.07^{a}	ND	0.06±0.01^{a}	0.51±0.05^{a}	0.14±0.01^{a}	0.54±0.05^{a}
∑SFA	40.46±4.05^{ab}	41.12±4.13^{a}	45.55±4.56^{a}	36.75±3.67^{b}	39.6±3.97^{bc}	36.08±3.61^{b}	42.09±4.23^{ab}	37.16±3.71^{b}	35.62±3.56^{b}	47.65±4.76^{a}	43.45±4.34^{a}	48.77±4.86^{a}
Monounsaturated												
14:1*n*-7	0.11±0.01^{a}	0.23±0.02^{a}	0.14±0.01^{a}	0.27±0.03^{a}	0.22±0.02^{a}	0.33±0.03^{a}	0.09±0.01^{a}	0.17±0.02^{a}	0.18±0.02^{a}	0.13±0.01^{a}	0.13±0.01^{a}	0.74±0.07^{a}
15:1*n*-7	0.08±0.01^{a}	0.12±0.01^{a}	0.13±0.01^{a}	0.08±0.01^{a}	0.09±0.01^{a}	0.21±0.02^{a}	0.19±0.02^{a}	0.1±0.01^{a}	0.2±0.02^{a}	0.09±0.01^{a}	0.11±0.01^{a}	0.74±0.07^{a}
16:1*n*-7	10.4±1.04^{a}	2.22±0.22^{b}	11.7±1.17^{a}	6.19±0.62^{c}	6.67±0.67^{c}	6.44±0.64^{c}	9.69±0.98^{a}	7.52±0.75^{ac}	10.2±1.02^{a}	5.42±0.54^{bc}	5.68±0.58^{bc}	5.12±0.51^{bc}
18:1*n*-7	0.13±0.01^{a}	0.29±0.03^{a}	0.05±0.01^{a}	0.46±0.05^{a}	0.43±0.04^{a}	0.43±0.04^{a}	0.47±0.05^{a}	0.13±0.01^{a}	0.13±0.01^{a}	0.43±0.04^{a}	0.32±0.03^{a}	0.51±0.05^{a}
18:1*n*-9	17.4±1.73^{a}	8.96±0.9^{b}	14.6±1.45^{a}	13.9±1.39^{a}	10.9±1.09^{ab}	13.6±1.35^{a}	8.6±0.86^{b}	7.59±0.76^{b}	8.84±0.87^{b}	15.9±1.6^{a}	13.8±1.38^{a}	16.2±1.62^{a}
20:1*n*-9	0.39±0.04^{a}	0.31±0.03^{a}	0.55±0.06^{a}	0.61±0.06^{a}	0.9±0.09^{a}	0.29±0.03^{a}	0.61±0.06^{a}	0.5±0.05^{a}	0.33±0.03^{a}	0.77±0.08^{a}	0.82±0.08^{a}	0.54±0.05^{a}
22:1*n*-9	1.95±0.2^{a}	4.31±0.44^{a}	1.91±0.19^{a}	3.43±0.34^{a}	3.71±0.37^{a}	2.62±0.26^{a}	2.52±0.25^{a}	2.37±0.24^{a}	1.77±0.18^{a}	3.58±0.36^{a}	4.34±0.43^{a}	3.56±0.36^{a}
24:1 *n*-9	0.33±0.03^{a}	1.56±0.16^{a}	0.36±0.04^{a}	0.54±0.05^{a}	0.26±0.03^{a}	1.06±0.11^{a}	0.35±0.04^{a}	0.4±0.04^{a}	0.22±0.02^{a}	0.32±0.03^{a}	0.09±0.01^{a}	0.25±0.03^{a}
∑MUFA	30.79±2.07^{a}	18±1.81^{b}	29.44±0.94^{a}	25.48±1.55^{a}	23.18±3.32^{abc}	24.98±2.48^{ac}	22.52±1.27^{ab}	18.78±1.88^{b}	21.87±0.17^{bc}	26.64±0.67^{a}	25.29±2.53^{a}	27.66±1.76^{a}
Polyunsaturated												
16:2*n*-4	0.14±0.01^{a}	0.23±0.02^{a}	0.3±0.03^{a}	0.45±0.05^{a}	0.29±0.03^{a}	0.2±0.02^{a}	0.26±0.03^{a}	0.57±0.06^{a}	0.18±0.02^{a}	0.38±0.04^{a}	0.41±0.04^{a}	0.15±0.02^{a}
16:3*n*-4	0.19±0.02^{a}	0.35±0.04^{a}	0.44±0.04^{a}	0.29±0.03^{a}	0.32±0.03^{a}	0.2±0.02^{a}	0.22±0.02^{a}	0.33±0.03^{a}	0.24±0.02^{a}	0.09±0.01^{a}	0.21±0.02^{a}	0.21±0.02^{a}
18:2*n*-6 LA	1.13±0.11^{a}	1.25±0.12^{a}	1.39±0.14^{a}	1.1±0.11	1.56±0.16	1.4±0.14	1.32±0.13	1.69±0.18	2.33±0.23	1.22±0.12	1.24±0.12	1.82±0.19
18:3*n*-6	0.96±0.1^{a}	0.54±0.05^{a}	0.99±0.1^{a}	1.3±0.13^{a}	1.91±0.19^{a}	1.49±0.15^{a}	0.52±0.05^{a}	0.42±0.04^{a}	0.61±0.06^{a}	0.57±0.06^{a}	0.74±0.07^{a}	0.57±0.06^{a}
18:3*n*-3 ALA	0.25±0.03^{ab}	0.31±0.03^{ab}	0.26±0.03^{ab}	1.25±0.13^{a}	0.54±0.05^{ab}	0.25±0.03^{ab}	0.1±0.01^{b}	0.56±0.06^{ab}	0.52±0.05^{ab}	0.3±0.03^{ab}	0.14±0.01^{ab}	0.25±0.03^{ab}
18:4*n*-3	ND	ND	0.88±0.09	ND	ND	0.08±0.01	ND	ND	ND	0.01±0	0.05±0.01	0.13±0.01
20:2*n*-6	0.48±0.05^{a}	0.52±0.05^{a}	0.15±0.02^{a}	1.02±0.1^{a}	0.72±0.07^{a}	0.46±0.05^{a}	1.16±0.12^{a}	1.7±0.17^{a}	1.67±0.17^{a}	1.11±0.11^{a}	0.58±0.06^{a}	1.08±0.11^{a}
20:3*n*-6	0.09±0.01^{a}	0.1±0.01^{a}	0.33±0.03^{a}	0.42±0.04^{a}	0.3±0.03^{a}	0.33±0.03^{a}	0.09±0.01^{a}	0.25±0.03^{a}	0.09±0.01^{a}	0.24±0.02^{a}	0.2±0.02^{a}	0.35±0.04^{a}
20:4*n*-6	0.61±0.06^{a}	0.33±0.03^{a}	0.76±0.08^{a}	0.68±0.07^{a}	0.37±0.04^{a}	0.48±0.05^{a}	0.34±0.03^{a}	0.55±0.06^{a}	0.52±0.05^{a}	0.34±0.03^{a}	0.49±0.05^{a}	0.36±0.04^{a}
20:5*n*-3 EPA	6.21±0.62^{a}	5.21±0.53^{a}	6.44±0.64^{a}	10.5±1.04^{b}	11.5±1.15^{b}	11.4±1.14^{b}	9.5±0.95^{b}	9.96±1^{b}	11.2±1.12^{b}	2.3±0.23^{a}	2.04±0.2^{a}	4.12±0.41^{a}
22:5*n*-3	2.45±0.24^{a}	2.02±0.2^{a}	0.56±0.06^{b}	1.33±0.13^{ab}	1.25±0.13^{ab}	1.31±0.13^{ab}	0.62±0.06^{b}	1.21±0.12^{ab}	0.56±0.06^{b}	0.85±0.09^{ab}	0.61±0.06^{a}	0.79±0.08^{ab}
22:6*n*-3 DHA	13.4±1.34^{a}	25.8±2.58^{b}	5.12±0.51^{c}	6.18±0.62^{c}	7.14±0.71^{c}	6.36±0.64^{c}	6.48±0.64^{c}	9.56±0.96^{ac}	7.24±0.72^{c}	7.24±0.73^{c}	7.52±0.75^{c}	6.35±0.64^{c}
∑PUFA	25.91±1.59^{a}	36.66±3.66^{b}	17.62±1.77^{cd}	24.52±2.45^{a}	25.9±0.59^{a}	23.96±2.41^{ac}	20.61±2.05^{ac}	26.8±0.71^{a}	25.16±2.51^{a}	14.65±1.47d	14.23±1.41d	16.18±0.65^{cd}

Data are expressed as mean ± standard deviation of three replicates. All other notations are as indicated in Table 3.1.

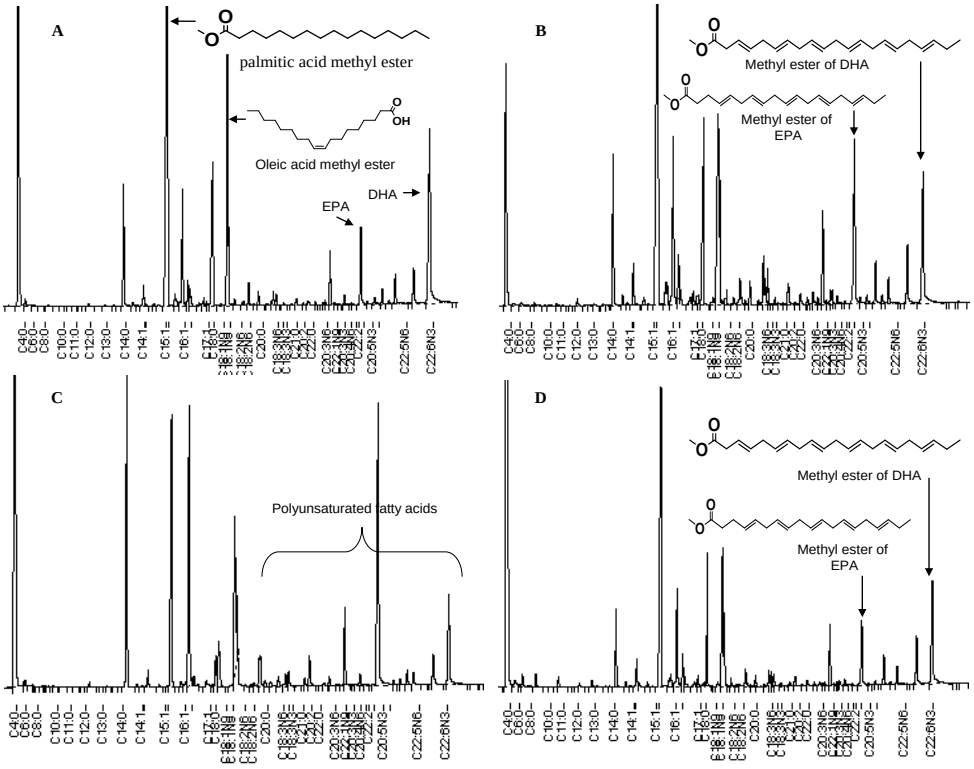


Fig. 3.3. Indicative chromatograms of fatty acid composition in (**A**) *Trichiurus lepturus*; (**B**) *Leiognathus splendens*; (**C**) *Sardinella longiceps* and (**D**) *Epinephelus diacanthus*

3.2.2D. Fatty Acid Based Nutritional Indices of Candidate Marine Fishes Collected from Southwest and Southeast Coasts of India

Fatty acid based nutritional Indices of candidate marine fishes are given in Table 3.2. *n*-3/*n*-6 ratio is a marker of biomedical significance for fish oils and could be an index of biomedical application. The mean *n*-3/*n*-6 fatty acid ratio recorded maximum value for ribbon fishes collected from SE coast of India (7.5), followed by oil sardines/silver bellies (4.4) and groupers (3.0) (Table 3.2). Along SW coast, ribbon fishes collected from SW coast of India recorded maximum *n*-3/*n*-6 fatty acid ratio (5.4), followed by oil sardines (5.1), silver bellies or groupers (3.0). Oil sardines collected from SW coast possessed maximum *n*-3/*n*-6 fatty acid ratio during pre-monsoon while all other species recorded maximum *n*-3/*n*-6 value during post-monsoon. Though insignificant, the difference in the *n*-3/*n*-6 ratio between different seasons in the present study may be explained by the large variability of the lipid content of the fish, which depends on the species, period of the year, age, size, reproduction period, the specific species as well as the fatty acid composition of the

diet. The *n*-3/*n*-6 fatty acid ratio is a good index for comparing the relative nutritional value of fish, and thus it is important for human health to increase the consumption of *n*-3 fatty acids. It has been reported that a high dietary consumption of marine *n*-3 fatty acids may prevent the development of atherosclerosis and thrombosis (Calder 2006). An increase in the human dietary *n*-3/*n*-6 fatty acid ratio is useful to prevent coronary heart disease by reducing plasma lipids and to reduce the risk of cancer (Kinsella *et al.*, 1990). Even though there are diversified metabolic functions of *n*-3 and *n*-6 PUFAs, the right balance between them is recommended. Earlier reports stated that high levels of *n*-6 PUFA in the human diet results in many health disorders, while *n*-3 PUFA have been reported to alter the adverse effects of *n*-6 PUFA (Ramesh *et al.*, 2013). An increase in the human dietary *n*-3/*n*-6 fatty acid ratio is essential to help prevent coronary heart disease by the reducing plasma lipids and to reduce cancer risk.

The *n*-6/*n*-3 ratio has been suggested to be a useful indicator for comparing the relative nutritional value of a given fish. The mean *n*-6/*n*-3 fatty acid ratio recorded highest value for silver bellies collected from SW coast of India (0.28) followed by groupers (0.27), oil sardines (0.2) and ribbon fishes (0.19). Similarly, groupers collected from SE coast of India recorded maximum *n*-6/*n*-3 fatty acid ratio (0.33), followed by oil sardines/silver bellies (0.3) and ribbon fishes (0.17). According to the UK Department of Health, an *n*-6/*n*-3 ratio within 0.20 - 1.50 would constitute a healthy human diet, and values higher than 1.50 would be harmful, and may promote cardiovascular diseases (Wijendran & Hayes 2004).

The mean PUFA/SFA ratio observed to be highest in the oil sardines (0.84) followed by silver bellies (0.69), ribbon fishes (0.6) and groupers (0.47) collected from the SW coast of India. However, along SE coast of India, maximum mean PUFA/SFA ratio was observed in silver bellies (0.66), oil sardines (0.64), ribbon fishes (0.6) and groupers (0.32). The recommended threshold value of the PUFA / SFA ratio for a healthy diet is 0.45 (HMSO 2001). The fishes collected from SW coast far exceed the minimum required PUFA/SFA ratio, and, are therefore, considered to be a health food.

A high DHA/EPA ratio was observed for ribbon fishes collected from both SW and SE coast of India (3.1 & 2.6, respectively) and the lowest DHA/EPA ratio was observed for oil sardines (0.4) collected from SW coast and silver bellies collected from SE coast (0.59). Hossain (2011) reported that a higher ratio of

DHA/EPA has an advantageous impact on consumer health and reducing the risk of coronary heart diseases.

A high LA/ALA ratio was observed for ribbon fishes collected from SW coast (5.2) and oil sardines collected from SE coast (6.9). In human nutrition, 18:2*n*-6 and 18:3*n*-3 are regarded as essential since they cannot synthesize by organism. The human body can synthesize all the PUFAs from the two precursors the ALA (18:3*n*–3) and the LA (18:2*n*–6) by two different enzymes elongase and desaturase (Wanasundara & Shahidi 1999). Vertebrates lack Δ^{12} and Δ^{15} (*n* - 3) fatty acid desaturases and cannot produce LA and ALA from oleic acid (Zlatanos & Laskaridis 2007). In the present investigation the LA/ALA ratio observed to be lower in SW coast, especially during monsoon season (0.8).

The atherogenicity (AI) and thrombogenicity indices (TI) are the lipid quality indicators that depend on the comparative contents of a particular set of fatty acids, which point out the global dietetic value of lipids. As a result, their potential effect on the development of coronary diseases (Jankowska *et al.,* 2010) was calculated. The AI & TI were found to be higher for all the fishes collected from the SE coast than those from the SW coast. Among them, groupers recorded highest AI and TI indices ($\geq$ 1.3 & $\geq$ 0.66, respectively) and the lowest AI and TI indices were observed for oil sardines and silverbellies collected from the SW coast ($\leq$ 1.0 & $\leq$ 0.4, respectively). The higher *n*-3 fatty acid content and consequently the higher *n*-3/*n*-6 fatty acid ratio in the oil sardines from the SW coast apparently contributed to lower AI and TI indices for the samples from the SW coast. The comparatively lower values of AI and TI correlated well with the lower SFA content of oil sardines collected during the monsoon season. Further, lower AI and TI of the oil sardines highlights their anti-atherogenic and anti-thrombogenic potential. These results provided us with the valuable information regarding oil sardine to be qualified as an ideal health food. It has been reported that due to the anti-atherogenic and anti-thrombogenic properties, the *n*-3 PUFAs play a major role to protect human beings from atherosclerosis and platelet aggregation (Barrento *et al.,* 2010). The HH ratio observed to be maximum for silver bellies (1.78 – 1.83) and oil sardines (1.4 -2.1) collected from the SW coast of India. The ideal HH ratio along with low AI and TI noted in the oil sardines also contributed towards its qualities to be judged as desirable from the consumer health perspective.

Table 3.2 Fatty acid based nutritional indices of *S. longiceps* collected from Southwest and Southeast coast of India during pre-monsoon, monsoon and post-monsoon seasons

	Trichiurus lepturus			*Leiognathus splendens*			*Sardinella longiceps*			*Epinephelus diacanthus*		
	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon	Pre-monsoon	Monsoon	Post-monsoon
SOUTH WEST COAST												
EPA + DHA	14.69±1.47^{a}	19.27±1.93^{b}	21.46±2.05^{b}	14.81±1.48^{a}	16.69±1.69^{ab}	16.07±1.59^{ab}	18.06±1.8^{b}	21.32±2.12^{b}	23.45±2.35^{b}	11.66±1.16^{a}	13.57±1.36^{a}	14.65±1.48^{a}
∑*n*-3 PUFA	16.11±1.62^{a}	22.77±2.18^{b}	22.63±2.17^{b}	16.49±1.65^{a}	19.48±1.98^{ab}	18.41±1.82^{ab}	19.54±1.95^{ab}	25.27±2.51^{b}	25.88±2.59^{b}	13.01±1.29^{a}	15.45±1.54^{a}	19.74±2.01^{ab}
∑*n*-6 PUFA	2.69±0.28^{a}	5.33±0.54^{ab}	3.74±0.38^{ab}	4.54±0.46^{ab}	6.81±0.69^{b}	3.7±0.37^{ab}	3.52±0.35^{ab}	5.64±0.56^{ab}	4.95±0.49^{ab}	3.8±0.39^{ab}	4.26±0.44^{ab}	4.66±0.47^{ab}
∑n-3/∑n-6	5.99±0.79^{ab}	4.27±0.04^{ab}	6.05±5.71^{a}	3.63±1.59^{ab}	2.86±1.87^{b}	4.98±1.92^{ab}	5.55±2.57^{ab}	4.48±1.48^{ab}	5.23±0.29^{ab}	3.42±0.31^{ab}	3.63±1.5^{ab}	4.24±1.28^{ab}
∑n-6/∑n-3	0.17±0.17^{a}	0.23±0.25^{a}	0.17±0.18^{a}	0.28±0.28^{a}	0.35±0.35^{a}	0.2±0.2^{a}	0.18±0.18^{a}	0.22±0.22^{a}	0.19±0.19^{a}	0.29±0.3^{a}	0.28±0.29^{a}	0.24±0.23^{a}
∑PUFA/∑SFA	0.42±0.42^{a}	0.73±0.71^{a}	0.65±0.63^{a}	0.66±0.65^{a}	0.76±0.77^{a}	0.65±0.64^{a}	0.69±0.7^{a}	0.99±0.97^{a}	0.84±0.85^{a}	0.38±0.38^{a}	0.43±0.43^{a}	0.59±0.6^{a}
LA/ALA	6±0.5^{a}	1.13±1.15^{b}	8.33±7.5^{a}	1.89±0.2^{b}	1.49±1.42^{b}	2.3±2.4^{b}	2.17±2.33^{b}	0.66±0.67^{b}	2.4±2.22^{b}	3.76±4.33^{ab}	1.95±2.17^{b}	0.91±0.79^{b}
AI	0.98±0.97^{a}	0.71±0.57^{a}	0.9±1.87^{a}	0.63±1.54^{a}	0.65±1.31^{a}	0.6±1.5^{a}	0.6±1.37^{a}	0.86±1.62^{a}	1±1.85^{a}	1±0.03^{a}	0.97±2.2^{a}	0.82±1.74^{a}
TI	0.61±0.35^{a}	0.4±0.34^{a}	0.43±0.3^{a}	0.41±0.33^{a}	0.4±0.35^{a}	0.4±0.3^{a}	0.37±0.26^{a}	0.29±0.23^{a}	0.34±0.25^{a}	0.73±0.56^{a}	0.65±0.5^{a}	0.49±0.37^{a}
HH ratio	1.27±1.28^{a}	1.61±1.58^{a}	1.39±1.36^{a}	1.8±1.8^{a}	1.83±1.86^{a}	1.78±1.74^{a}	1.48±1.47^{a}	2.11±2.09^{a}	1.4±1.41^{a}	0.98±0.99^{a}	0.98±0.99^{a}	1.27±1.26^{a}
SOUTH EAST COAST												
EPA + DHA	19.61±1.96^{a}	31.01±3.11^{b}	11.56±1.15^{c}	16.68±1.66^{ac}	18.64±1.86^{a}	17.76±1.78^{a}	15.98±1.59^{ac}	19.52±1.96^{a}	18.44±1.84^{a}	9.54±0.96^{c}	9.56±0.95^{c}	10.47±1.05^{c}
∑*n*-3 PUFA	22.31±0.23^{a}	33.34±0.34^{b}	13.26±1.33^{c}	19.26±0.92^{a}	20.43±2.04^{a}	19.4±1.95^{a}	16.7±1.66^{ac}	21.29±0.14^{a}	19.52±1.95^{a}	10.7±1.08^{c}	10.36±1.03^{c}	11.64±1.17^{c}
∑*n*-6 PUFA	3.27±0.33^{a}	2.74±0.26^{a}	3.62±0.37^{a}	4.52±0.45^{a}	4.86±0.49^{a}	4.16±0.42^{a}	3.43±0.34^{a}	4.61±0.48^{a}	5.22±0.52^{a}	3.48±0.34^{a}	3.25±0.32^{a}	4.18±0.44^{a}
∑n-3/∑n-6	6.82±0.76^{a}	12.17±1.85^{b}	3.66±0.59^{a}	4.26±1.27^{a}	4.2±1.16^{a}	4.66±2.64^{a}	4.87±1.88^{a}	4.62±1.46^{a}	3.74±0.75^{a}	3.07±1.18^{a}	3.19±1.22^{a}	2.78±0.66^{a}
∑n-6/∑n-3	0.15±0.15^{a}	0.08±0.08^{a}	0.27±0.28^{a}	0.23±0.23^{a}	0.24±0.24^{a}	0.21±0.22^{a}	0.21±0.2^{a}	0.22±0.22^{a}	0.27±0.27^{a}	0.33±0.31^{a}	0.31±0.31^{a}	0.36±0.38^{a}
∑PUFA/∑SFA	0.64±0.64^{a}	0.89±0.89^{a}	0.39±0.39^{a}	0.67±0.67^{a}	0.65±0.65^{a}	0.66±0.67^{a}	0.49±0.48^{a}	0.72±0.73^{a}	0.71±0.71^{a}	0.31±0.31^{a}	0.33±0.32^{a}	0.33±0.34^{a}
LA/ALA	4.52±3.67^{a}	4.03±4^{a}	5.35±4.67^{a}	0.88±0.85^{b}	2.89±3.2^{a}	5.6±4.67^{a}	13.2±13^{c}	3.02±3^{a}	4.48±4.6^{a}	4.07±4^{a}	8.86±12^{ac}	7.28±6.33^{ac}
AI	0.62±0.62^{a}	0.67±0.67^{a}	1.36±1.36^{a}	0.74±0.75^{a}	0.79±0.79^{a}	0.71±0.72^{a}	1.36±1.36^{a}	1.17±1.15^{a}	1.1±1.11^{a}	1.2±1.19^{a}	1.06±1.06^{a}	1.05±1.04^{a}
TI	0.42±0.25^{a}	0.3±0.16^{a}	0.73±0.48^{a}	0.4±0.27^{a}	0.44±0.3^{a}	0.42±0.27^{a}	0.57±0.35^{a}	0.43±0.29^{a}	0.43±0.3^{a}	0.84±0.53^{a}	0.82±0.51^{a}	0.84±0.58^{a}
HH ratio	1.32±1.32^{a}	1.53±1.53^{a}	0.79±0.79^{a}	1.53±1.52^{a}	1.43±1.42^{a}	1.56±1.56^{a}	0.84±0.83^{a}	1.11±1.12^{a}	1.18±1.17^{a}	0.84±0.85^{a}	0.89±0.88^{a}	0.91±0.92^{a}

Data are expressed as mean ± standard deviation of three replicates. *n*-3 means total *n*-3 fatty acids; *n*-6 means total *n*-6 fatty acids; 20:5*n*-3 = EPA; 22:6*n*-3 = DHA; ∑SFA Total saturated fatty acids; ∑PUFA =Total polyunsaturated fatty acids; 18:2*n*-6 = Linoleic acid; 18:3*n*-3 = α-linolenic acid; AI = atherogenicity index; TI = Thrombogenicity index; HH ratio = hypocholesterolemic/hypercholesterolemic ratio; Different superscripts (a, b etc) in the same row indicate statistical difference ($p<0.05$).

3.2.3. Nutritional Composition of *Sardinella longiceps* Collected from Southwest coast of India

The present study demonstrated that *Sardinella longiceps* is exceptionally rich in the essential PUFAs, especially EPA and DHA. The dominance of the essential fatty acids in SW coast collected oil sardines is clearly evident from this study. The high lipid content and significantly low amount of saturated fatty acids with good amount of EPA, DHA, other essential *n*-3 PUFAs and nutritional indices make the oil sardines collected from SW coast of India superior over other studied marine fishes. Hence the seasonal variation in the overall nutritional qualities with respect to proximate composition, true protein, amino acids, vitamins and minerals of the *Sardinella longiceps* collected from SW coast of India was further studied.

3.2.3.1. Proximate Composition of *Sardinella longiceps* Collected from Southwest coast of India

The proximate composition of the *Sardinella longiceps* collected from SW coast of India during three different seasons is recorded in Table 3.3. No seasonal differences in the length of sardines were observed ($p>0.05$) while the sardines collected during monsoon season (48.2 g) weighed more compared to pre-monsoon and post-monsoon samples (35.3 & 27.7 g) ($p<0.05$). The moisture content observed post-monsoon maxima (72.2 %) and showed minimum value during pre-monsoon season (67.5 %). The crude protein was found to be maximum during monsoon (19.4 %) and minimum during post-monsoon (16.5 %). The variations in the protein and fat composition are closely related to the feed intake of the fish. During periods of heavy feeding, the protein content of muscle tissue increases slightly at first and then the fat content might show a marked and rapid increase. On the other hand, fish may have starvation periods for natural or physiological reasons (spawning or migration) or because of external factors such as shortage of food. In that case, fat content gradually decreases and then a decline in protein may also be seen (Boran & Karaçam 2011). The average ash content recorded was maximum for the samples collected during monsoon (2.8 %) and minimum for the samples collected during

post-monsoon (1.2 %). In comparison with other proximate components crude fiber was found to be low in all the seasons.

Table 3.3 Proximate composition of *Sardinella longiceps* collected from Southwest coast of India

	Pre-monsoon	Monsoon	Post-monsoon
Length (cm)	13.8±0.02^{a}	15.2±1.52^{a}	13±1.65^{a}
Weight (g)	35.3±0.53^{a}	48.2±1.82^{b}	27.7±0.98^{c}
Moisture (%)	67.5±1.75^{a}	68.9±1.94^{a}	72.2±2.2^{a}
Crude Protein (%)	17.1±1.75^{a}	19.4±2.85^{a}	16.5±1.65^{a}
Crude Fat (%)	9.1±0.84^{a}	9.6±0.6^{a}	8.4±0.8^{a}
Crude Ash (%)	2.1±0.22^{a}	2.8±0.25^{a}	1.2±0.23^{a}
Crude Fiber (%)	0.07±0.007^{a}	0.06±0.006^{a}	0.03±0.003^{a}

Data are expressed as mean ± standard deviation of three replicates. Different superscripts (a-c) in the same row indicate statistical difference ($p<0.05$).

3.2.3.2. True Protein Content and Amino Acid Composition in *Sardinella longiceps* Collected from the Southwest coast of India during Monsoon Season

The true protein content in oil sardines collected from SW coasts is shown in Table 3.4. Proteins were found to be an essential nutritional component in *S. longiceps*, and are essentially required by the human beings for growth and survival. The total protein content ranged from 11.2 - 19.3 g/100g in SW coast samples. The current study indicated substantial seasonal variation in the true protein content with maximum value during pre-monsoon along SW coast (19.3 g/100g). This variation in the total protein content can be attributed to the feeding of different protein rich marine phytoplanktons and to climatic changes, which influence the general biochemical composition of the fish. In addition, as no gonadal elements are present in pre-monsoon, the food that is consumed is used in the building up of the muscle. During the peak spawning season, i.e., during monsoon in coasts the protein content was low compared to pre-monsoon because during spawning period the fishes has been found to be active and agile and this results in the utilization of some of the muscle reserves of energy results in decline of muscle protein. In general, the seasonal profile of the protein from oil sardines collected from SW coast illustrated the active growing phase during monsoon and a decrease after spawning, in post-monsoon.

The essential, non-essential amino acid compositions of *S. longiceps* from SW coast are recorded in Table 3.4, respectively. The amino acid profile of oil sardines from SW coast showed that all the essential amino acids were significantly higher in concentrations, when compared with the reference pattern (FAO/WHO 1990), which implied that the proteins present had a high biological value, and are therefore called complete proteins. The oil sardine protein contains a broad variety of amino acids and their isomers especially high proportion of the essential amino acids (EAA) which were about 50 - 59 % TAA. The percentage ratios of TEAA to TAA (TEAA/TAA) in the samples were higher than 50 % which are well above the 39 % considered to be adequate for ideal protein food for infants, 26 % for children and 11 % for adults (FAO/WHO/UNU, 1985). The major dominated EAA in the protein content of the sardines from were valine, arginine, leucine, lysine etc. The EAA content was observed maximum during monsoon (5.0 g/100g) followed by post-monsoon (4.5 g/100g) dominating valine (1.3 g/100g), phenylalanine (1.3 g/100g) and lysine (0.62 g/100g). An increase in the serine level, an amino acid in the human body which assists the function of the central nervous system was observed maximum during monsoon (0.88). Interestingly, oil sardines collected during monsoon and post-monsoon seasons possess high lysine content which is severely restricted in cereals, the most important staple food in the world. Concerning non-essential amino acid (NEAA), the major ones found in oil sardines were glutamic acid, glycine, serine, cysteine with maximum NEAA observed during monsoon (4.0 g/100g), followed by post-monsoon (3.1 g/100g) and pre-monsoon (2.8 g/100g). The TEAA/TNEAA ratio which observed more than 1.0 during the monsoon and post-monsoon seasons which indicated that oil sardine in these seasons could provide high quality proteins or well-balanced protein deposition. Any ratio of TEAA/TNEAA amino acids higher than 1.0 is considered to be excellent, and therefore it can be concluded that oil sardines during the monsoon and post-monsoon are sources of well balanced proteins and high-quality protein source in respect of TEAA/TNEAA ratio. The TEAA/TNEAA ratio observed by Iwasaki and Harada (1985) was considerably lower for other marine species like *Pagrus major* (0.77), *Scomber japonicus* (0.77), *O. keta* and *Paralichthys olivaceus* (0.77) compared to the present

study. TEAA/TNEAA ratio observed maximum during post-monsoon (1.4) where as TEAA/TAA ratio observed post-monsoon maxima (0.59), whereas high TNEAA/TAA ratio was observed maximum during pre-monsoon (0.5) and TArAA showed monsoon maxima (2.0 g/100g). TSAA (cysteine + methionine) showed higher values in monsoon (1.0 g/100g). Cys:TSAA ratio also showed higher values in monsoon (0.9), while leucine: isoleucine ratio showed post-monsoon maxima (1.5 g/100g). Arg:Lys ratio observed maximum values in pre-monsoon (1.1 g/100g).

Table 3.4 Protein (g/100g edible portion) and amino acid composition (g/100g edible portion) of *S. longiceps* collected from Southwest coast of India during three different seasons (pre-monsoon, monsoon and post-monsoon)

	Pre-monsoon	Monsoon	Post-monsoon
Protein (g/100g)	19.3±1.93^{a}	16.5±1.65^{a}	11.2±1.12^{b}
Histidine (His)^{p} (1.9 mg/100g)	0.17±0.02^{a}	0.33±0.03^{a}	0.51±0.05^{a}
Arginine(Arg)^{p}	0.58±0.06^{a}	0.25±0.03^{a}	0.71±0.07^{a}
Threonine^{p}(Thr) (3.4 mg/100g)	0.25±0.03^{a}	0.21±0.02^{a}	0.32±0.03^{a}
Valine^{p}(Val)(3.5 mg/100g)	0.31±0.03^{a}	1.32±0.13^{a}	0.41±0.04^{a}
Methionine^{p}(Met)	0.18±0.02^{a}	0.09±0.01^{a}	0.19±0.02^{a}
Isoleucine^{p}(Ileu) (2.8 mg/100g)	0.27±0.03^{a}	0.65±0.07^{a}	0.39±0.04^{a}
Leucine^{p}(Leu) (6.6 mg/100g)	0.26±0.03^{a}	0.23±0.02^{a}	0.57±0.06^{a}
Phenylalanine^{p}(Phe)	0.26±0.03^{a}	1.3±0.13^{b}	0.68±0.07^{ab}
Lysine^{p}(Lys) (5.8 mg/100g)	0.51±0.05^{a}	0.62±0.06^{a}	0.75±0.08^{a}
Alanine(Ala)^{q}	0.41±0.04^{a}	0.21±0.02^{a}	0.5±0.05^{a}
Cysteine(Cys)^{q}	0.03±0^{a}	0.95±0.1^{a}	0.02±0^{a}
Glutamic acid(Glu)^{q}	0.94±0.09^{a}	1.24±0.12^{a}	1.15±0.12^{a}
Glycine(Gly)^{q}	0.52±0.05^{a}	0.09±0.01^{a}	0.33±0.03^{a}
Proline(Pro)^{q}	0.37±0.04^{a}	0.21±0.02^{a}	0.24±0.02^{a}
Serine(Ser)^{q}	0.38±0.04^{a}	0.88±0.09^{a}	0.34±0.03^{a}
Tyrosine(Tyr)^{q}	0.13±0.01^{a}	0.42±0.04^{a}	0.54±0.05^{a}
TEAA	2.79±0.28^{a}	5±0.5^{a}	4.53±0.45^{a}
TNEAA	2.78±0.28^{a}	4±0.4^{a}	3.12±0.31^{a}
TAA	5.57±0.56^{a}	9±0.9^{b}	7.65±0.77^{ab}
TEAA/TAA	0.5±0.05^{a}	0.56±0.06	0.59±0.06
TNEAA/TAA	0.5±0.05^{a}	0.44±0.04^{a}	0.41±0.04^{a}
TEAA/TNEAA	1±0.1^{a}	1.25±0.13^{a}	1.45±0.15^{a}
TArAA	0.56±0.06^{a}	2.05±0.21^{b}	1.73±0.17^{b}
TSAA	0.21±0.02^{a}	1.04±0.1^{b}	0.21±0.02^{a}
Arg:Lys	1.14±0.11^{a}	0.4±0.04^{a}	0.95±0.1^{a}
Leu: Ileu	0.96±0.1^{a}	0.35±0.04^{a}	1.46±0.15^{a}
Cys: TSAA	0.14±0.01^{a}	0.91±0.09^{a}	0.1±0.01^{a}

^{p}Essential amino acids; ^{q}Non-essential amino acids; TEAA- Total amino acids; TNEAA – Total non-essential amino acids ; TAA - Total amino acids ; TArAA - Total aromatic amino acids; TSAA - Total sulphur containing amino acids; Data are expressed as mean ± standard deviation (n = 3); Different superscripts (a, b etc) within a row denote significant differences ($p<0.05$). FAO/WHO reference pattern (1990) for evaluating proteins (mg/ 100g) were indicated in parentheses (FAO/WHO, 1990). Tryptophan was not determined.

3.2.3.3. Vitamin and Mineral Content in *Sardinella longiceps* Collected from the Southwest coast of India

The vitamin and mineral content of *S. longiceps* collected from SW coast of India are shown in Table 3.5. The vitamin A content was significantly higher during post-monsoon (8.2 µg/100g) coast compared with pre-monsoon and monsoon (≤ 5.0 µg/100g). The vitamin D content in the sardines collected from was significantly higher in monsoon (862 µg/100g). Vitamin D is essential for the maintenance of normal blood levels of Ca and phosphate (Trivedi *et al.*, 2003). A pre-monsoon maximum (1.3 µg/100g) was observed for vitamin E content, while vitamin K observed post-monsoon maxima (3.6 µg/100g). Vitamin E acts as an antioxidant against peroxidation of fatty acid contained in the cellular and sub cellular membrane phospholipids leading to the formation of phenoxy free radicals. Vitamin K plays an important role in blood clotting and bone metabolism pertaining to the prevention of osteoporosis and carotid artery elasticity. Apparently, vitamin C content was observed monsoon maxima (12.6 µg/100g, respectively). Vitamin C is an essential nutrient for humans, but an additional external dietary source is required because it is not synthesized by human metabolism (Jeevitha *et al.*, 2013.).

The macro (Na, K, Ca and P) and micro (Fe, Mn, Zn, Se) mineral concentrations during the three seasons from SW coast of India are depicted in Table 3.5. A high Na/K ratio was observed during post-monsoon for samples (0.8), which can be associated with an increased risk of developing high blood pressure and cardiovascular diseases. The concentrations of alkaline metals (Na, K, Ca) and phosphorus were significantly higher during monsoon. The values of Ca+P ratio were found to be maximum during monsoon (662 mg/100g). Food is considered "good" if the Ca/P ratio ≥ 1.0 and "poor" if the ratio ≤ 0.5. The oil sardines collected during both monsoon and post-monsoon seasons observed Ca/P ratio more than 1.0 during monsoon season. All the micro minerals (Fe, Mn, Zn) were also observed to be maximum during monsoon season (650, 80 & 5750 µg/100g, respectively). Zn was the most abundant micro element followed by Fe and Mn during all the seasons studied. Saadettin *et al*. (1999) reported that the most abundant microelements in fish

were Zn and Fe followed by Cu with the remaining elements present in amounts below toxic levels. Mn content in all the samples was found to be lower than the permissible limit set by FAO/WHO (1984) 5.4 ppm or 540 µg/100g foods. Similarly, the oil sardines contained Zn lower than the limit set by FAO/WHO (1984) (150 ppm or 15000 µg/100g). The abundance of Fe, Mn and Zn was likely to be due to high bioavailability of these elements arising in the fishes by a high metal absorption from the food chain as a consequence of high feeding activity. Se concentration ranged from 10 – 20 µg/100g, and maximum value was observed during post-monsoon season. The Se content in oil sardine obtained in oil sardines were significantly higher (10 - 20 µg/100g) than cereals (<10 µg/100g), fruits and vegetables (<10 µg/100g) (Levander *et al*., 1994) and observed to be maximum during post-monsoon.

Table 3.5 Vitamin and mineral composition of *S. longiceps* collected from Southwest coast of India during three different seasons (pre-monsoon, monsoon and post-monsoon)

	Pre-monsoon	Monsoon	Post-monsoon
Vitamins			
Vit A	4.2 ± 0.12^{a}	5.0 ± 0.06^{a}	8.2 ± 0.09^{b}
Vit D_3	458.0 ± 1.25^{a}	862.0 ± 0.25^{b}	212.0 ± 0.02^{c}
Vit E	1.3 ± 0.12^{a}	0.9 ± 0.09^{a}	0.1 ± 0.001^{a}
Vit K	0.2 ± 0.003^{a}	1.0 ± 0.05^{a}	3.6 ± 0.13^{a}
Vit C	11.2 ± 0.22^{a}	12.6 ± 0.23^{a}	5.5 ± 0.22^{b}
Macro minerals			
Na	62.60 ± 0.03^{a}	112.6 ± 0.23^{b}	97.89 ± 0.94^{b}
K	125.3 ± 0.14^{a}	265.5 ± 2.13^{b}	122.9 ± 3.53^{a}
Na/K	0.50 ± 0.02^{a}	0.42 ± 0.05^{a}	0.80 ± 0.08^{a}
Ca	143.5 ± 0.45^{a}	369.3 ± 0.74^{b}	186.7 ± 1.05^{c}
P	458.6 ± 1.13^{a}	292.5 ± 0.24^{b}	178.5 ± 1.03^{c}
Ca/P	0.3	1.3	$1\pm$
Ca+P	602.1 ± 0.25^{a}	661.8 ± 0.14^{a}	365.2 ± 1.24^{b}
Micro minerals			
Fe	260 ± 1.4^{a}	650 ± 1.05^{b}	450 ± 2.03^{c}
Mn	20.0 ± 0.03^{a}	80.0 ± 0.15^{b}	20.0 ± 0.13^{a}
Zn	3230 ± 1.15^{a}	5750 ± 1.03^{b}	1990 ± 1.18^{c}
Σmicro	3510 ± 2.06^{a}	6480 ± 124^{b}	2460 ± 2.03^{a}
Se (µg/100g)	10 ± 0.11^{a}	$10\pm .23^{a}$	20 ± 0.05^{b}

Vitamin A, D_3, E, K and C are represented in µg/100g. Data are expressed as mean ± standard deviation (n = 3). Different superscripts (a-c) within a column denote significant differences ($p<0.05$). Data are expressed as mean ± standard deviation (n = 3); Different superscripts (a-d) within a column denote significant differences ($p<0.05$). Macro minerals (mg/100g edible portion) are Na, K, Ca and P; Micro minerals (µg/100g edible portion) are Fe, Mn, and Zn.

3.3. Conclusions

The present study demonstrated that *Sardinella longiceps* is a valuable source of long-chain PUFAs, especially, EPA and DHA, for human diet as well as the extraction of oil in view of commercial exploitation. It has been demonstrated that *S. longiceps* harvested from the Southwest coast, especially during monsoon/post-monsoon, showed its superiority over those harvested from the Southeast coast when EPA, DHA, total polyunsaturated fatty acids and all other fatty acid based nutritional indices were considered. Compared to the oil sardines collected during post-monsoon, insignificant variations in fatty acid based health indices, higher lipid yield, maximum EPA content and low saturated fatty acids etc were observed for the oil sardines collected during the monsoon season (Southwest coast). This demonstrated the importance of this low-value fish collected during monsoon season from the Southwest coast for commercial use, particularly for marine fish oil industries. This study also showed that *S. longiceps* collected from the Southwest coast during monsoon season is a source of ideal nutrition due to the optimum balance of the essential nutrients such as amino acids, minerals and vitamins. Therefore, it can be concluded further that the *S. longiceps* collected during the monsoon season from Southwest coast can be considered as ideal raw material for further extraction, refinement and purification of essential PUFAs as described in the following chapters.

Chapter 4

EXTRACTION AND REFINING OF TRIACYLGLYCERIDES FROM *SARDINELLA LONGICEPS*

Contents

Background

The crude sardine oil consists primarily of TAGs that occur as storage fat in hydrophobic aggregates, which contain fatty acids of varying chain lengths and degree of unsaturation. In order to increase the industrial application and utilization of fats and oils from the marine origin, recovery procedures that can give high yield of oil extracted without compromising the oil quality are crucial. As per those results, a good extraction technique was to be standardized to obtain a substantially pure PUFA extract, which can be suggested for use in nutritional and clinical purposes. Thus, this chapter aimed to investigate the effect and influence of different extraction methodologies on the yield of the lipid content and other lipid quality parameters. Six different extraction techniques were attempted to acquire the crude oil from the edible tissues of *Sardinella longiceps*, and were compared by evaluating their fatty acid composition and broad-spectrum quality markers such as specific gravity, free fatty acid (FFA) value, saponification value (S.V.), iodine value (I.V.), thiobarbituric acid reactive species value (TBARS), peroxide value (P.V.), para-anisidine value (pA.V.), total oxidation value (TOTOX), and oxidative stability index (OSI) using rancimat analysis.

Despite the presence of valuable functional compounds, mainly long chain PUFAs in the crude lipids, it contains glycerides, free fatty acids, phospholipids, sterols, tocopherols, pigments and insoluble impurities that curtail its quality. Therefore, the crude oil requires refining treatment to remove these undesirable components to produce high quality oils suitable for human consumption. As a

result, this chapter further designed to optimize the operational conditions of the refining processes of the crude oil, which include degumming (removal of phospholipids/wax esters), neutralization of the free fatty acids with alkali (to clear free fatty acid and reduce oil acidity), bleaching with different adsorbents (to adsorb contaminants and pigments), and deodorization by vacuum distillation (to remove low molecular weight volatile compounds such as aldehydes and ketones). Refining aims to improve the quality of sardine oil by removing the impurities. Each stage of these refining processes (Fig. 4.1) was followed by evaluating the quality parameters, as described above. The degree of change in the color of the oil during different stages of refining have been estimated using Lovibond CIE-L*a*b* color space. The different stages of refining were monitored using spectral analyses such as ultraviolet-visible (UV-VIS) absorption Gas chromatography-mass spectrometry (GC-MS) and Nuclear magnetic resonance spectrometry (NMR) experiments.

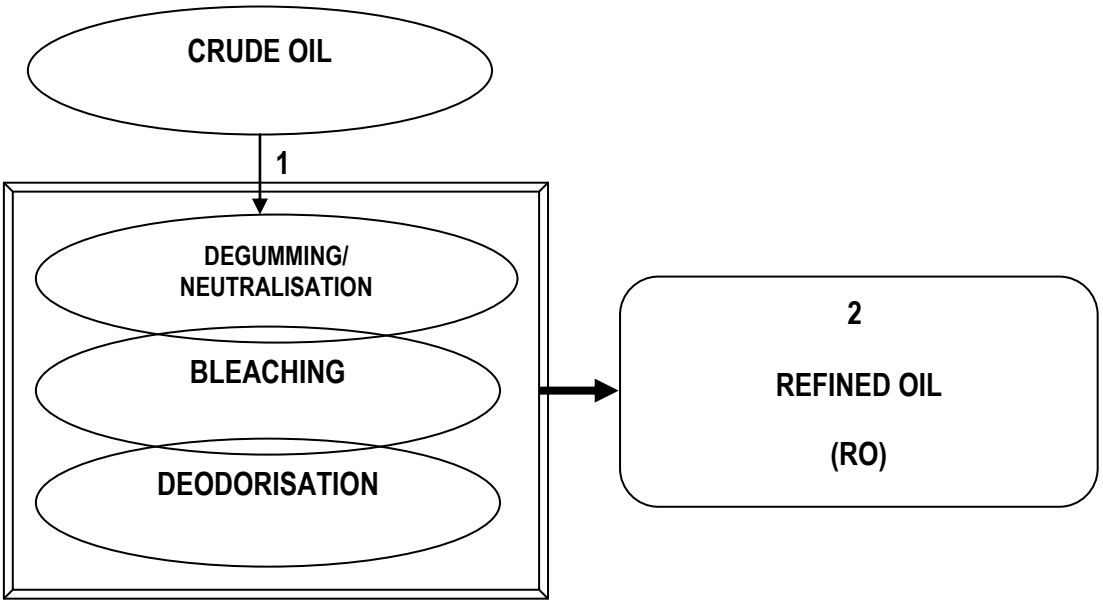


Fig. 4.1. A schematic diagram showing the overall refining process (1) – Crude oil obtained after extraction with optimum selected extraction method was refined by degumming, neutralization, bleaching and deodorization to get the refined oil (2)

4.1. Materials and Methods

4.1.1. Chemicals, Reagents and Instrumentation

The chemicals/reagents such as NaOH, KOH, HCl, Kaolin light, Fuller's Earth, Cellulose powder, Chitin purified powder, NaOH, glacial acetic acid, oxalic acid, citric acid, phosphoric acid, deionized water, Na_2SO_4, activated charcoal powder, silica gel, phenolphthalein, CCl_4, Wij's solution, potassium thiosulphate, potassium iodide, sodium hydroxide solution, potassium iodide, sodium thiosulphate, malondialdehyde (MDA), 2-thiobarbituric acid (TBA), and *p*-anisidine were procured from E-Merck (Germany) or HiMedia (India). The solvents such as $CHCl_3$, MeOH, EtOH, $CHCl_3$, hexane, toluene, isopropyl alcohol etc were purchased from E-Merck (Germany), and were of analytical grade.

A screw expeller (40 – 50 ^oC) PIC-106 (Four Brothers Ex - Imp Pvt Ltd., Chennai) were used for pressing of the crude oil from sardines. A Lovibond colorimeter (Lovibond color staler tintometer, model F, England) was used for finding the CIE-L*a*b* color space. A Metrohm Rancimat model 743 (Metrohm Instruments, Herisau, Switzerland) was utilized for studying the oxidative stability of the sardine oil. P.V. was conducted using a Titrino Plus 848 automatic titrator (Metrohm Instruments, Herisau, Switzerland). For UV-VIS spectroscopic analysis, an UV-VIS Spectrophotometer (Varian Cary, USA) was used. GC-MS analysis of extracted oil was performed in a Varian GC (CP-3800) and the identification of the volatile compounds obtained in deodorization process was carried out by GC-MS (Perkin Elmer Clarus 680). FTIR analysis was performed in Thermo Avtar 370 FTIR spectrometer. NMR spectra were measured on a JEOL-AL 300 MHz instrument, using $CDCl_3$ as aprotic solvent at ambient temperature with tetramethylsilane (TMS) as the internal standard (δ 0 ppm). The ultra sonicator (Labline, India) was used for sonicating and a laboratory shaker (Labline, India) was used for shaking. A rotary vacuum evaporator (Heidolf, Germany) was used for evaporation of solvents.

4.1.2. Comparison of Different Extraction Modes of Crude Sardine Oil

The crude oil from the edible tissues of *Sardinella longiceps* were obtained in parallel by six different extraction methods. Six different methodologies carried out to obtain crude sardine oil from S. longiceps were as follows: (E_1) extraction using Bligh and Dyer method ($CHCl_3$: MeOH, 2:1, v/v), (E_2) extraction using aqueous ethyl alcohol (EtOH 40% v/v), (E_3) extraction using commercial ethyl alcohol (EtOH 95% v/v) as extracting solvent systems, (E_4) extraction using deionized water, (E_5) cooking followed by extraction using Bligh and Dyer method, and (E_6) cooking followed by pressing.

In Bligh and Dyer method (E_1), the minced edible tissues of oil sardines (100 g) were sonicated with a solvent mixture of $CHCl_3$: MeOH (2:1, 600 ml) for 10 min. A volume of about 80 ml of distilled water was added to this mixture, shaken at 150 rpm for 5 min (Shaker, Labline), and further sonicated for 5 min (Ultrasonicator, Labline). The homogenate was subsequently filtered *in vacuo*, the filtrate collected and transferred to a separatory funnel for phase separation. The lower organic fraction containing the lipid was filtered through a Whatman No.1 filter paper on a Buchner funnel and evaporated *in vacuo* by using a rotary vacuum evaporator.

In E_2, E_3 and E_4 methods, the minced edible tissue of oil sardines (100 g) was extracted twice with 40% EtOH, 95% EtOH and 100% distilled water (250 ml x 2), respectively. These extractions (E_2, E_3 and E_4) were performed by shaking the mixture at 150 rpm for 20 min, and further sonication for 10 min. The homogenate obtained after centrifugation for 30 min at 8,000 g (Thermo Scientific, Biofuge Stratos, Germany) was subsequently filtered through a Whatman No.1 filter paper to afford the filtrate containing the crude lipids. The filtrate containing the solvents or water was evaporated *in vacuo* by using a rotary vacuum evaporator (Heidolf, Germany) to obtain the crude sardine oil (E_2, E_3 and E_4).

In another method, the minced edible tissues of oil sardines (100 g) were mixed with deionized water (100 ml) and cooked in a pressure cooker for 30 min at 75 ^{o}C. The residual wet tissue obtained after cooking was centrifuged (8,000 g, 30

min), and further extracted by Bligh and Dyer method to obtain the crude sardine oil (E_5).

In E_6, the cooked wet tissue was pressed in a screw expeller (PIC-106; Four Brothers Ex - Imp Pvt Ltd., Chennai) prior to centrifuge (8,000 g, 30 min) to afford the cooked and pressed crude sardine oil (E_6).

The weight of the crude oil or lipid obtained was gravimetrically determined, and expressed as % w/w of the wet edible tissue. The final traces of solvents were removed by flushing with N_2.

The crude oils were studied for their quality parameters such as specific gravity, free fatty acid (FFA) value, saponification value (S.V.), iodine value (I.V.), thiobarbituric acid reactive species value (TBARS), peroxide value (P.V.), para-anisidine value (pA.V.), total oxidation value (TOTOX), oxidative stability index (OSI) using rancimat analysis, and fatty acid composition by GLC using authentic fatty acid standards (Sigma, St. Louis, MO). The oil obtained by optimum methodology of extraction was further assayed by GC-MS, ^{1}H –NMR, ^{13}C-NMR, $DEPT_{135}$ and FTIR spectral analysis.

4.1.3. Refining of Crude Sardine Oil

The crude oil obtained using pressing method (ie. E_6) was refined using degumming, neutralization, saponification, washing, bleaching and deodorization. The methodologies were detailed in the following sections. The term ‘‘neutralized’’ oil refers to the oil that has been degummed and neutralized; ‘‘bleached’’ oil refers to the oil that has been degummed, neutralized and bleached; and ‘‘deodorized’’ oil refers to the oil that has been degummed, neutralized, bleached and deodorized.

4.1.3.1. Degumming and Neutralization of Crude Sardine Oil

Crude sardine oil (E_6, 100 g) was stirred in a temperature-controlled water bath (70 ^{o}C) for 20 min separately with 85% phosphoric acid (1 ml, DG_1), 99% glacial acetic acid (1 ml, DG_2), 10% oxalic acid (5 ml, DG_3), and 10% citric acid (5 ml, DG_4). The oil was then cooled to room temperature and centrifuged (10,000 g, 10 min) to remove the precipitated gum. The optimum method of degumming was

found out by studying the percent recovery and common physico-chemical characteristics of the oil. The ^{1}H NMR analysis of the degummed oil was performed to study the effect of degumming in the crude sardine oil, and thereby the optimum reagent for effective degumming was selected. The proton integral as observed by the ^{1}H-NMR analysis of the degummed oil *vis-à-vis* crude oil were compared to understand the reduction of the hydrocarbon residues from the externalities.

The degummed oil (100 g) obtained by optimum degumming method (i.e., with phosphoric acid DG_1) was further refined by caustic neutralization according to the AOCS Official Method Ca 9b-52 (AOCS 1989). This was performed by the addition of NaOH solution (1N, 30 ml) drop wise to the degummed oil with constant stirring and heating (65 ^{o}C) for 20 min until a pH of 7.0 was attained. This oil was then heated at 70 ^{o}C for 20 min, cooled to room temperature, centrifuged (10,000 g, 10 min), and the oil was decanted to remove the precipitated soap. The oil thus obtained was washed thrice with 10 % deionized water (in relation to the oil mass) by agitation (500 rpm) and heating at 50 ^{o}C under vacuum. Water and other impurities were further removed by centrifuging (2,500g, 10 min) to obtain the neutralized sardine oil (NSO).

4.1.3.2. Bleaching of Neutralized Sardine Oil

The neutralized sardine oil obtained above (*section 4.1.3.1*; $B_{0;}$ control) was treated separately with activated charcoal powder (AC), kaolin light (KL), Fuller's earth (FE; fulmont 700C), cellulose powder (CP), and chitin purified powder (CH) to study the individual effect of different adsorbents (at 5%, w/w of oil) on the color and other quality parameters of the oil. The effect of different experimental combinations (at 5% w/w of oil) of the best adsorbents selected, (i.e., AC and FE) on bleaching the oil was further evaluated. The five experimental combinations of AC and FE were: (BC_1) AC + FE (90:10), (BC_2) AC + FE (75:25), (BC_3) AC + FE (50:50), (BC_4) AC + FE (25:75), and (BC_5) AC + FE (10:90). The best adsorbent combination was optimized by studying their effects on the oil quality parameters (FFAs, P.V., pA.V., TOTOX, TBARS value, Lovibond L*, a*, b* color, chroma, hue angle, ΔE values) after bleaching. For the above bleaching processes, the neutralized

oil was stirred separately with the adsorbents at 300 rpm (using a magnetic stirrer) with heating at 60 °C for 10 min under N_2. The effect of the best adsorbent combination, i.e., (BC_4), AC + FE (25:75) was further studied for optimizing two experimental factors, namely, adsorbent amount and contact time of bleaching as shown in Table 4.1. The bleached sardine oil obtained by the optimized bleaching condition, i.e., [(BC_{4E}; adsorbent amount (3%) and contact time (40 min)], was further assayed by ^{1}H NMR analysis.

Table 4.1 Adsorbent amount and contact time of bleaching

Treatments	Adsorbents		Amount of adsorbent (g in	Contact time (min)
	Activated charcoal (g)	Fuller's Earth (g)		
BC_{4A}	1.25	3.75	5	20
BC_{4B}	1.25	3.75	5	40
BC_{4C}	1.25	3.75	5	60
BC_{4D}	0.75	2.25	3	20
BC_{4E}	0.75	2.25	3	40
BC_{4F}	0.75	2.25	3	60
BC_{4G}	0.25	0.75	1	20
BC_{4H}	0.25	0.75	1	40
BC_{4I}	0.25	0.75	1	60

Adsorbent amount 5% - Activated Charcoal (1.25 g) + Fuller's earth (3.75 g)
Adsorbent amount 3% - Activated Charcoal (0.75 g) + Fuller's earth (2.25 g)
Adsorbent amount 1% - Activated Charcoal (0.25 g) + Fuller's earth (0.75 g)
Contact time implies the time used for stirring the oil with the adsorbent combination by heating at 60 °C.

4.1.3.3. Deodorization of Bleached Sardine Oil

The deodorization of the bleached sardine oil (BC_{4E}) was carried out using a modified method of Karahadian and Lindsay (1990) with a laboratory distillation unit containing a round bottom boiling flask (500 ml) having two outlets. One outlet was connected to a condenser, which was connected to vacuum, and other was connected to a thermometer. Aqueous acetic acid solution (0.25N, 5 ml) was added with aliquot

containing the BSO in a round-bottomed flask, and the content was heated to 100 ^{o}C under vacuum (5 mm Hg) for a period of one hour. The vacuum line was turned off at different time intervals (5, 15, 30, and 60 min), and the distillates (designated as DO_1 through DO_4, respectively) were analyzed using GC-MS to estimate the traces of volatile compounds distilled off during deodorization process. The traces of volatile compounds distilled off during deodorization process of the degummed oil were analyzed at different intervals (5, 15, 30 and 60 min) using gas chromatographic-mass spectroscopic (GC-MS) analysis. For removal of residual water and acids, when desired, the deodorized oils were passed through the beds of powdered anhydrous sodium sulfate/sodium bicarbonate (2:1, w/w) under reduced pressure (500 mm Hg), before being dried by passing N_2.

4.1.4. Analytical Methodologies for Oil Quality Parameters

The quality of the oil during extraction/refining processes was evaluated by determining several parameters as described in the following sections.

4.1.4.1. Specific Gravity

The specific gravity of the oil was measured using specific gravity bottle according to earlier described method (IAFMM, 1981). The specific gravity was calculated as: weight of bottle + oil – weight of bottle)/ weight of water at 25 ^{o}C.

4.1.4.2. Free Fatty Acid Value

The percentage of free fatty acids (FFAs) in each sample was determined by the titration method as described in AOCS method Ca 5a-40 (AOCS 1998). Briefly, the oil was weighed into a flask followed by neutralization by using 95% ethyl alcohol and a phenolphthalein indicator. The mixture was then titrated against sodium hydroxide solution (0.1 M) until a permanent pink color persisted for at least 30 s. Each sample was titrated in triplicate. This was expressed as the quantity of milligrams of potassium hydroxide (KOH) requisite to neutralize the FFA present in one gram of fat. FFA values were reported as % oleic acid by weight and measured by the following formula. FFA as % oleic acid = (28.2 X 0.1 X volume of 0.1N NaOH in ml) *100 / weight of oil in g.

4.1.4.3. Saponification Value

The degree of hydrolysis (%) was determined by measuring saponification value of oil according to AOCS method (1998). The saponification value is the amount of alkali necessary to saponify a definite quantity of the sample. It is expressed as the number of milligrams of potassium hydroxide required to saponify one gram of the sample. To determine the saponification value, a weighed sample of the fat was treated with a measured volume of alcoholic KOH solution and saponified by heating for about one hour. The excess of KOH was titrated with dilute HCl by using phenolphthalein as an indicator. A blank determination is run simultaneously with the sample and the titration values are calculated.

Saponification value, S.V (mg KOH/g) = [56.1(B-S) x N]/W
Where, B = Volume of standard HCl (ml) required for the blank
S = Volume of standard HCl (ml) required for the sample
N = Normality of standard HCl
w = Weight of the oil (g) taken for analysis

4.1.4.4. Iodine Value

Iodine value is an empirical test indicating the degree of unsaturation of fats or oils. Iodine value is defined as the number of grams of iodine absorbed by hundred grams of oil. The iodine value was determined according to the AOAC (2000), by using CCl_4 as the solvent. The dissolved oil sample was mixed with Wij's solution and freshly prepared potassium iodide (KI, 10%) solution. The liberated iodine (I_2) was titrated with standard potassium thiosulphate (0.1 M) solution, using CCl_4 as a blank and starch as an indicator.

Iodine value (g of I_2/100g of sample) = (Blank Titration – Sample Titration) x N of sodium thiosulphate) x 12.69 / weight of sample in g.

4.1.4.5. Peroxide Value (P.V.)

The peroxide value of the oils was determined in order to quantify the recent oxidation suffered by the oil. The peroxide value is a titration measure of the peroxides and lipid oxidation products that would oxidize potassium iodide. The peroxide value was determined using an automatic titrator according to the modified

method of AOAC official method 965.33 (AOAC 1990). The oil samples (2 g) were dissolved in the solvent mixture of $CHCl_3$ and glacial acetic acid (40:60, v/v). The freshly prepared saturated potassium iodide solution (0.5 ml) and deionized water (30 ml) were then added and the liberated iodine was titrated with standard sodium thiosulphate (0.01 M) solution. The blank determination was also carried out. The P.V. was expressed as meqO_2/kg oil.

4.1.4.6. The para-Anisidine Value (pA.V.)

The para (p)-anisidine value (pA.V.) analysis was estimated to measure the concentration of secondary oxidation products such as aldehydes, ketones and alcohols produced by decomposition of hydroperoxides. The p-anisidine value of the oil sample was determined by using the procedure described in the AOCS official method (AOCS 1995). Briefly, about 0.7 g of oil was placed into a 25 ml volumetric flask. It was dissolved and diluted to 25 ml with iso-octane. The absorbance was measured at 350 nm with spectrophotometer. In a test tube, 5 ml of iso-octane and 1 ml of p-anisidine reagent were added and used as blank. To another test tube, 5 ml of oil was transferred, and 1 ml of p-anisidine reagent was added. The absorbance was measured at 350 nm after 10 minutes. The p-anisidine value was expressed as: p-A.V. = [25 X (1.2 A_S - A_B)]/W; where: A_S = absorbance of the oil solution after reaction with the p-anisidine reagent, A_B = absorbance of the oil solution, W = grams of sample, 25 ml = dilution volume, 1.2 = correction factor.

4.1.4.7. Total Oxidation (TOTOX) Value

TOTOX value was computed to evaluate the rancidity level of the oils, reflecting total oxidation to date. The total oxidation can be used to measure the progression of deterioration that occurs in oil and provide information on the formation of primary and secondary oxidation products (Hamilton & Rossell 1986). TOTOX is a comprehensive oxidation index calculated from a weighted sum of P.V. and pA.V. (Shahidi & Wanasundara 2002) and calculated as, TOTOX= (2 x P.V.) + pA.V.

4.1.4.8. Thiobarbituric Acid Reactive Species (TBARS) Value

The thiobarbituric acid-reactive substances (TBARS) assay was performed as described by Buege and Aust (1978). The fish oil (0.5 ml) was mixed with 2.5 ml of the TBA solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl. The mixture was heated in boiling water for 10 min to develop a pink color, cooled with running tap water, and then sonicated for 30 min followed by centrifugation at 5,000 g at 25 °C for 10 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1, 1, 3, 3-tetramethoxypropane (malondialdehyde; MDA) at a concentration ranging from 0 to 10 ppm, and TBARS was expressed as mg of MDA equivalents (MDAEQ)/kg oil.

4.1.4.9. Rancimat Analysis

The oxidative stability of oils was determined by measuring their induction time (IT) by rancimat method as described by Yi *et al*. (1991) with modifications. In brief, the sardine oil (3 g) was subjected to accelerated oxidative conditions in a rancimat instrument at an airflow rate of 20 l/h and a temperature of 80 °C. The conductivity measuring cells contained 70 ml of distilled water. The induction time (IT) was automatically determined as the inflection point of the generated plot of conductivity (μS/cm) of the water versus time (h). Analyses were performed in triplicate. The oil in the presence and absence of antioxidants were oxidized under the rancimat conditions, and the index of antioxidant activity (AAI) was estimated according to Nwosu *et al*. (1997) as: AAI = IT with antioxidant/IT without antioxidant.

4.1.4.10. Lovibond Color

The color of the sardine oil during degumming, neutralization and bleaching process was determined using a Lovibond Colorimeter based on the method of Shativel *et al*. (2003). The results were expressed as L*, a*, and b* values. L* values measure lightness (0 = black and 100 = white), whereas a* values represent redness (+) and greenness (-), and b* values represent yellow (+) and blue (-). From a* and b*, the psychometric parameters hue-angle (h_{ab}; actual color) and chroma (C^*_{ab};

intensity of color) were calculated as, $h_{ab} = \tan^{-1} (b^*/a^*)$ and $C^*_{ab} = \sqrt{(a^*)^2 + (b^*)^2}$. Total color difference was calculated by $(\Delta E) = \sqrt{(\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})}$. The hue-angle is the attribute according to which colors have been traditionally defined as reddish, greenish and others (Wyszecki & Stiles 1967). The chroma value is related to the quantitative attribute of colorfulness, and allowed determining (for each hue-angle) the degree of difference when compared to a grey color with the same lightness.

4.1.4.11. Ultraviolet Absorption

The ultra violet spectra for the treated and control sardine oils were determined on a Varian Cary UV-VIS Spectrophotometer equipped with a 1 cm quartz cell. The UV spectral scanning was performed at a wavelength range of 340 nm – 400 nm for a drop of oil sample added to *n*-hexane and made up to 5 ml in a standard flask. The absorbance was measured as: $A = \log (I_0/I) = Ecl$, where, C= concentration of sample (moles per litre); e = molar extinction coefficient, A= absorbance at a particular wavelength, l= path length of the light through the sample (cm).

4.1.4.12. Fatty Acid Profiling

The fatty acid composition of the oil after converting to FAMEs, after various extraction modes and different stages of refining were evaluated using gas liquid chromatography (GLC) as explained in *Chapter 3*.

4.1.4.13. Gas Chromatography-Mass Spectrometry (GC/MS) analysis of fatty acid methyl esters (FAMEs) derived from optimum extraction mode

The GC-MS analyses were performed using the electronic impact (EI) ionization mode in a Varian GC (CP-3800) interfaced with a Varian instrument 1200L single quadruple mass spectrometer for confirmation of fatty acid identification. The FAMEs were derivatized to N-acyl pyrrolidides by condensation of fatty acid methyl ester with a mixture of pyrrolidine (1 ml) and acetic acid (0.1 ml) at 100 °C under reflux (2 h) for GC-MS analyses (Andersson 1978). The GC apparatus was equipped with WCOT fused silica capillary column of high polarity (DB-5; 30 m x 0.25 mm i.d., 0.39 mm o.d., and 0.25 µm film thickness; Varian). The

polymeric stationary phase was nonpolar (VF-5MS, 5% phenyl substituted methylsiloxane). The carrier gas was ultra-high purity He (99.99% purity) with a constant flow rate of 1 ml/min. The injector and detector temperature were maintained isothermal at 300 °C. The injection volume was 1 µl. Samples were injected in split (1:15) mode at 300 °C into the capillary column similar to that used for the GC analyses, and the oven was identically programmed. The ion source and transfer line were kept at 300 °C. Mass spectra were analyzed using Varian Workstation (version 6.2) software.

4.1.4.14. NMR Analysis

NMR spectra were measured on a JEOL-AL 300 MHz, using TMS (Tetramethylsilane) as an internal standard and $CDCl_3$ as a solvent (in δ ppm). The ^{1}H-NMR spectra were recorded on a Bruker ACP- 300 MHz NMR spectrometer. The concentration of the sample used was 5–10% w/w in CDCl3 for 1H NMR containing tetramethylsilane (TMS) as the internal reference under the following experimental conditions: spectral width = 5,000 Hz (0.0–12.0 ppm), spectral size = 16 K, digital resolution = 0.49 Hz/point, , relaxation delay = 10 s and number of scans = 64. The ^{1}H-NMR spectra were integrated three times after base line correction and a mean of three integration values was taken for each calculation.

4.1.4.15. FTIR (Fourier Transform Infrared Spectroscopy)

A small quantity of the sample was added to KBr in the ratio 1:100 approximately. The matrix was grinded for 3-4 minutes using mortar and pestle. The fine powder was transferred into 13 mm diameter die and made into a pellet using a hydraulic press by applying a pressure of 7 tonnes. The fine pellet was subjected to FTIR analysis by employing a universal pellet holder. (a single drop of oil is poured on the KBr pellet in case of liquid samples). Infrared spectral data were collected on a Thermo Avtar 370 FTIR spectrometer. Spectra are collected over a range of 4000–400 cm^{-1}at 4 cm^{-1} resolution with an interferogram of 32 scans.

4.1.4.16. GC-MS Analysis of the Volatile Components Obtained during Deodorization

The volatile component analysis was carried out by GC-MS (Perkin Elmer Clarus 680). Separation of volatile flavor was achieved on Elite 5 MS non-polar, bonded phase capillary column (50 m × 0.22 mm ID × 0.25 μm film thicknesses). Helium (He) was used as the carrier gas and the flow rate used was 1 ml/min. The temperature was programmed at an initial temperature of 50 °C for 2 min, then to 10 °C /min to 180 °C and held for 2 min. finally 4 °C /min to 280°C and held for 15 min. About 1μL of the extract was injected into the column, in the split-less mode. Electron ionization energy was set at 70 eV. The mass range, electron multiplier voltage and scan rates were set at 35-400 u, 1550 V and 10 spectra/second, respectively. Ion source temperature was maintained at 200 ºC.

4.1.5. Statistical Analysis

Statistical evaluation was carried out with the Statistical Program for Social Sciences 13.0 (SPSS Inc, Chicago, USA, ver. 13.0). Analyses were carried out in triplicate, and the means of all parameters were examined for significance by analysis of variance (ANOVA). The level of significance for all analyses was $p \leq 0.05$.

4.2. Results and Discussion

4.2.1. Comparison of Different Extraction modes of Crude Oil from *Sardinella longiceps*

4.2.1.1. Yield

The yield or recovery of the oil was similar ($p > 0.05$) for E_1 (8.5 %) and E_6 (8.3 %) (Table 4.2). Hence, the use of cooking followed by pressing method was found to be preferable while considering the use of carcinogenic solvent ($CHCl_3$) in the Bligh and Dyer method. The oil extracted from the Japanese Sardine with $CHCl_3$:MeOH (2:1, v/v) showed similar yield as in the present study (7.0 g/100 g wet weight) (Toyoshima *et al*., 2004). The use of polar solvents (E_3, 5.6 %; E_2, 2.8 % and

E_4, 2.7 %), as well as cooking followed by extraction with $CHCl_3$: MeOH (E_5, 6.9 %) exhibited lesser yield (Table 4.2) than E_1 and E_6. The lesser yield of E_2 and E_4 could be attributed to the limited efficiency of the highly polar water and ethyl alcohol in extracting non-polar lipids from the oil sardine tissues compared to $CHCl_3$: MeOH system. Due to its feeble non-polar character, 95 % ethanol (E_3) was able to extract more lipids when compared to 40% ethanol (E_2) and aqueous (E_4) extraction. Yang et al. (2014) obtained comparable lipid yield (33 %) during ethanol extraction with that of Bligh and Dyer method, from wet microalga *Picochlorum* sp. However, the present study revealed lesser lipid yield for ethanol extraction (5.6 %) than that obtained by Bligh and Dyer method (8.5 %). The lesser yields of E_2, E_3 and E_4 as compared to the cooking methods (as in E_5 and E_6) demonstrated the effect of cooking, which coagulated the protein of fish tissues and thereby easier separation of solids by centrifugation. In addition, the pre-cooking generated the expansion and breaking of cell structure that made it more permeable and improving the oil yield (Li *et al.*, 1999). A photograph of E_6 is shown in Fig. 4.2.

Fig.4.2. Crude sardine oil obtained by cooking followed by pressing (E_6)

4.2.1.2. Oil Quality Parameters

Table 4.2 presents the experimental values of different oil quality parameters. The obtained crude oil possessed differences in the quality parameters between the different extraction techniques.

Specific gravities of the crude sardine oil were found to be maximum for E_3 followed by E_4, E_1, E_5, E_6 and E_2, in descending order. The specific gravity of the oil

was found to be in the range 0.91 – 0.93 (Table 4.2), which was close to that of the standard value of 0.907 to 0.915 (Adeniyi & Bawa, 2006).

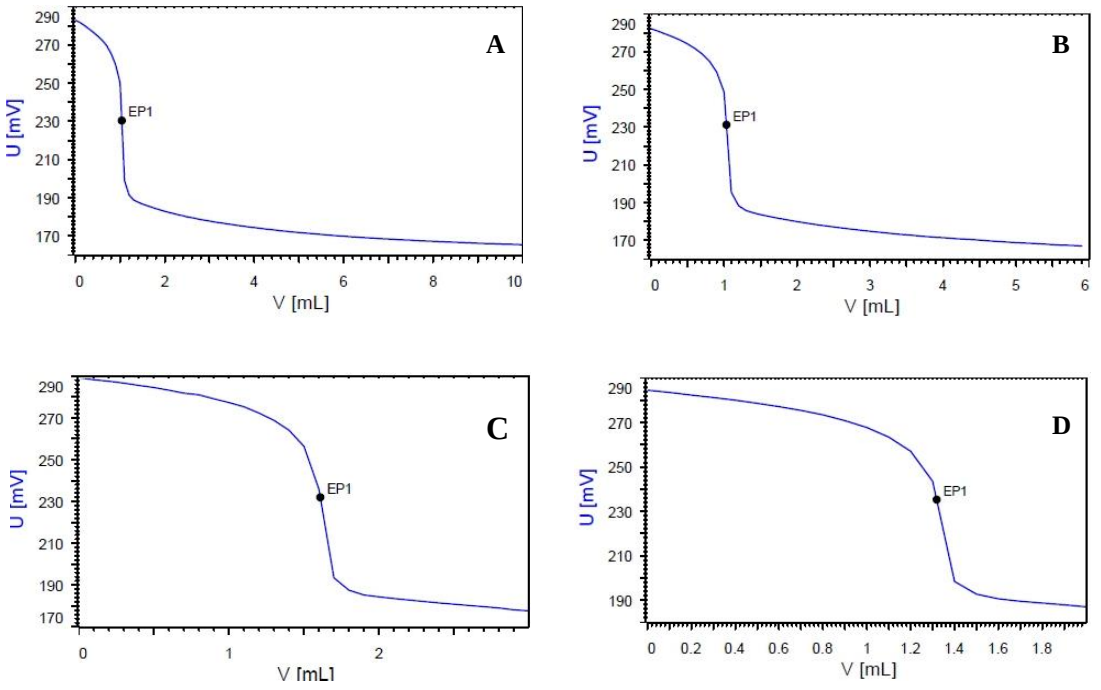
The FFA contents were usually associated with undesirable flavor and textural changes when they are present in the fats and oils. . In the oil processing industry, the FFA are determined for providing an indication of the amount of alkali to be used for their removal as soaps during the refinement process (Sathivel *et al*., 2003). The FFA value for different extraction methods was in the order, $E_4 > E_3 \sim E_5 > E_2 > E_1 > E_6$ (Table 4.2). A significantly greater level of FFA in E_1, E_2, E_3, E_4 and E_5 (> 6 % $p < 0.05$) and a comparable level of FFA in E_6 (4.4 % $p > 0.05$) to the acceptable limits of FFA (2- 5 %) (Bimbo 1998) was realized. The greater FFA content in E_1, E_2, E_3, E_4 and E_5 could be attributed due to the thermal process and the greater moisture content in these methodologies, which cause faster hydrolysis of the ester bonds in the triacylglycerols to free fatty acids (Irianto & Giyatmi 2009).

The S.V. of the crude fish oil was found to be greater for E_3 followed by E_5, E_2, E_1, E_4 and E_6, in descending order (Table 4.2). The S.V. in the present study was in the range 209 - 246 mg KOH/g, which was marginally greater than that of standard value for fish oil (180-200 mg KOH/g) (AOCS 1992). The significantly lesser ($p < 0.05$) S.V. of the oil obtained using cooking and pressing method (E_6) indicated the occurrence of higher molecular weight fatty acids as compared to the oils attained using other methods. The greater S.V. of the treatments E_1 - E_5 could be attributed to the presence of impurities, such as sterols, glyceryl ethers, hydrocarbons, fatty alcohols and some minor quantities of pigments and vitamins, present in the crude fish oil.

The iodine value (I.V.) reflects the degree of unsaturation of oil or fat and demonstrates the amount of unsaturation present in the sample. A greater I.V. was observed for E_3 (212 g I_2/100g) followed by E_4 (179 g I_2/100g), E_6 (170 g I_2/100g), E_1 (162 g I_2/100g), E_5 (154 g I_2/100g) and E_2 (118 g I_2/100g), in descending order (Table 4.2). Chantachum *et al*. (2000) observed I.V. in the range of 122 to 174 g I_2/100g for crude oil from the tuna heads. It is of note that the oil extracted using 95 % ethyl alcohol (E_3), H_2O (E_4) showed significantly greater ($p < 0.05$) iodine value

than the oil extracted by Bligh and Dyer method (E_1). The oil obtained by cooking followed by pressing method (E_6) showed similar iodine values to that of oil obtained using Bligh and Dyer method (E_1) ($p > 0.05$). This indicated that the extraction methodologies, E_3, E_4 and E_6 showed lesser oxidation of its double bonds than those observed in the Bligh and Dyer method (E_1).

The peroxide values of E_1 - E_6 are shown in Fig. 2A-F. The P.V. was found to be > 21 meqO_2/kg except for E_6 (8.4 meq O_2/kg) against the standard value of 3 - 20 meqO_2/kg (Bimbo 1998). The P.V. was found to be significantly lesser for E_6 (11.9 meqO_2/kg; $p < 0.05$) as compared to E_1- E_5, which followed the order: $E_1 < E_2 < E_5 < E_4 < E_3$ (Fig. 4.3A-F, Table 4.2). Interestingly, the oil obtained by E_1 showed greater yield (8.5 %), the P.V. of E_1 was found to be 22 meqO_2/kg. E_5 involved two heat treatments for cooking and distillation. These heat treatments might have increased the primary lipid oxidation (P.V. 29.3 meqO_2/kg) thereby generating hydroperoxides. Peroxide values followed the same trend as the FFA contents, which indicate that the deterioration occurred during the extraction process, not only owing to oxidation but also due to hydrolysis.



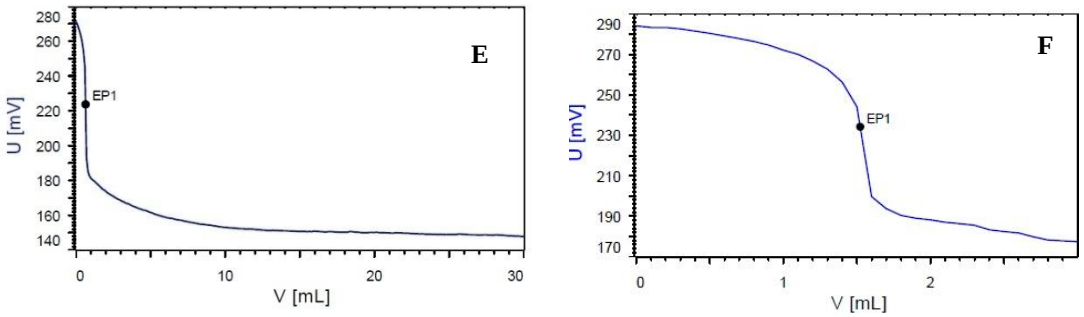


Fig. 4.3. Potentiometric titration curves obtained using automatic titrator indicating the peroxide levels in (**A**) E_1; (**B**) E_2; (**C**) E_3; (**D**) E_4; (**E**) E_5 (**F**) E_6

The *para*-anisidine value (pA.V.) showed greater values for E_1 followed by E_5, E_2, E_4, E_3 and E_6 (Table 4.2). The initial pA.V. of 18 - 36 was recorded to be significantly greater ($p < 0.05$) as compared to safflower oil (1.1), rapeseed (3.5) and olive oil (5.1) (Guillén & Cabo 2002). Slightly greater pA.V. values, although not insignificant ($p > 0.05$), were found for E_2 - E_5 as compared to the pre-cooked oil (E_6) due to the higher temperatures needed during the process of extraction and solvent removal, thereby causing the PUFA an oxidative damage and the resultant formation of undesirable components. However, pressing of the oil employed mild extraction conditions (low heat), which avoid potential alterations to the extracted oil.

The TOTOX value was found to be more for E_1 to E_5 (> 64) (Table 4.2) as compared to the standard range of 10-60 (Bimbo 1998). The pressing method (E_6) significantly reduced the TOTOX value as compared with other extraction methods (E_1 to E_5; $p < 0.05$). It is evident that E_6 showed significantly lower TOTOX value than those in E_1-E_5 ($p < 0.05$) owing to the lesser generation of hydroperoxides during cooking and pressing process.

Secondary lipid oxidation was studied by the determination of TBARS value, an index of malonaldehyde (MDA) concentration. No significant differences ($p > 0.05$) were noted in the TBARS value of E_2 - E_6 when compared with the standard Bligh and Dyer method (E_1) (Table 4.2). However, considering the greater degree of unsaturation found in E_6, the minimum value observed for TBARS (6.18 MDAEQ/kg) showed the importance of the extraction process using cooking and pressing.

The oxidative stability index is determined as a time point when the induction period characterized by low conductivity is interrupted by a rapid increase in conductivity as a result of accelerated oxidation. Rancimat induction period was expressed as resistance or induction time (in hours) of the oil to oxidation. The rancimat induction time of pre-cooked, pressed oil was greater (E_6 0.13 h) than in the oil extracted by other methodologies (E_1-E_5), which indicate the drawbacks of improper extraction methods such as aqueous and solvent extractions. The rancimat curves showing the induction (IT) of E_{1-6} are shown in Fig. 4.4A-F (Table 4.2). Berthiaume and Tremblay (2006) observed lesser ITs for fish oil ethyl ester (0.2 h), followed by yellow grease methyl ester (0.4 h), tallow methyl ester (0.5 h), soybean methyl ester (0.8 h) and canola methyl ester (1.3 h). However, Mendez et al. (1997) showed that partially refined sardine oil exhibited greater IT (1.5 h) at 80 ^oC than those in the present study, and it might be due to the additional refining step by the removal of oxidation products, pigments and off-flavour compounds. The greater content of PUFA, mainly EPA and DHA, in E_1 with respect to E_6 would explain the lowest oxidative stability in E_1 (IT 0.05 h).

Table 4.2 Characteristics of crude sardine oil obtained using different modes of extraction

	E_1	E_2	E_3	E_4	E_5	E_6
Lipid (%)	8.5 ± 0.85^a	2.81 ± 0.28^b	5.57 ± 0.56^{ab}	2.68 ± 0.27^b	6.89 ± 0.69^{ab}	8.31 ± 0.83^a
Specific gravity	0.916 ± 0.09^a	0.912 ± 0.09^a	0.927 ± 0.09^a	0.922 ± 0.09^a	0.914 ± 0.09^a	0.913 ± 0.09^a
FFA	6.2 ± 0.62^{ab}	8.3 ± 0.83^a	9.4 ± 0.94^a	10 ± 0.01^a	9.4 ± 0.94^a	4.4 ± 0.44^b
S.V.	222.6 ± 2.26^{ab}	234.5 ± 3.44^a	246.2 ± 2.62^a	213.6 ± 2.36^b	235 ± 2.5^a	209.8 ± 0.98^b
I.V.	162 ± 1.2^a	118 ± 1.7^b	212 ± 12.2^c	179 ± 1.5^{ac}	154 ± 1.1^a	170 ± 1.14^a
P.V.	21.97 ± 2.2^a	22.1 ± 2.21^a	28.33 ± 2.83^a	27.26 ± 2.73^a	29.3 ± 2.93^a	11.88 ± 1.19^b
pA.V.	35.61 ± 3.56^a	20.2 ± 2.02^b	18.36 ± 1.84^b	19.26 ± 1.93^b	23.31 ± 2.33^b	16.2 ± 1.62^b
TOTOX	79.55 ± 3.96^a	64.4 ± 2.44^a	75.02 ± 0.5^a	73.78 ± 1.38^a	81.91 ± 2.19^a	39.96 ± 0.01^b
TBARS	6.28 ± 0.63^a	6.33 ± 0.63^a	6.29 ± 0.63^a	8.45 ± 0.85^a	6.31 ± 0.63^a	6.18 ± 0.62^a
I.T	0.05	0.03	0.03	0.03	0.1	0.13

E_1 – Extraction using $CHCl_3$: MeOH (2:1); E_2 – Aqueous EtOH (40%); E_3 - EtOH (95%); E_4 - H_2O; E_5 Cooking/$CHCl_3$:MeOH (2:1); E_6 – Crude oil obtained by cooking and pressing. Data are expressed as mean ± standard deviation of three replicates. Means with different superscripts (a, b etc) in the column indicate statistical difference ($p<0.05$). FFA – Free fatty acid value represented in % oleic acid; S.V. – Saponification value represented in mg KOH/g; I.V – Iodine value represented in g of I_2/100g; P.V. – Peroxide value represented in meqO_2/kg; pA.V – p-anisidine value; TOTOX – total oxidation value (2 x P.V + pA.V.); TBARS – Thiobarbituric acid reactive species represented in mg MDA equivalent compounds/kg sample (MDAEQ/kg); IT – Induction time in h.

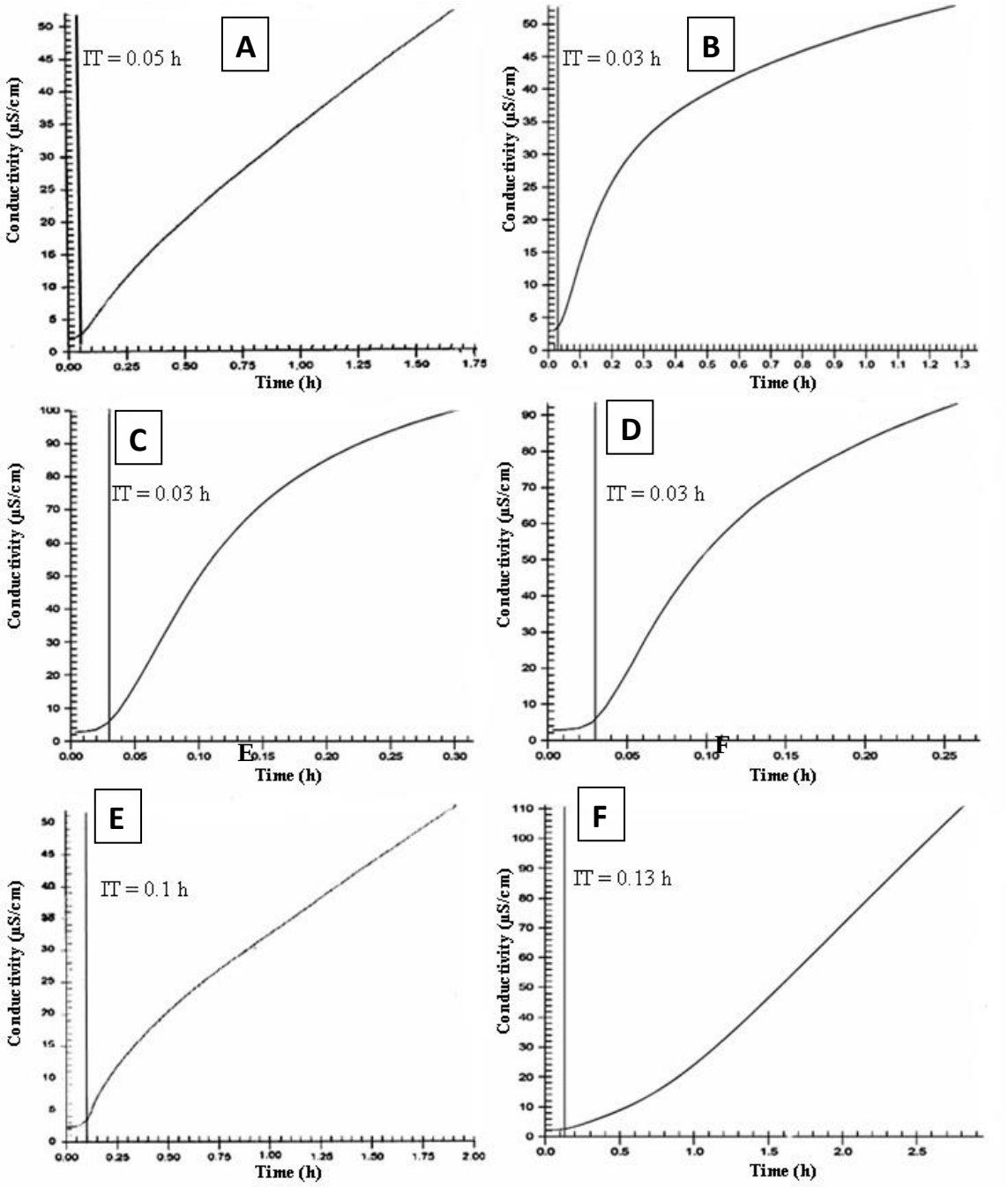


Fig.4.4. Rancimat curves showing the induction time (IT) indicating the stability index of (**A**) E_1; (**B**) E_2; (**C**) E_3; (**D**) E_4; (**E**) E_5 (**F**) E_6

4.2.1.3. Fatty Acid Composition of Crude Sardine Oil

The fatty acid compositions of the crude sardine oil obtained using various extraction modes were shown in Table 4.3. The SFA fractions of the oil extracted using cooking followed by Bligh and Dyer extraction was found to be maximum (E_5; 43.1 %), followed by E_3 (41.5 %), E_2 (41.3 %), E_6 (41 %), E_4 (40.8 %), and E_1 (30.6 %), in descending order. The predominant SFA in the different extraction modes was 16:0, accounting for more than 46% of total SFA content. The total PUFA obtained using E_1 and E_6 (30.8 and 26.5 %) were observed to be significantly greater than those obtained in E_2 through E_5 (< 22 %) ($p < 0.05$).

The total *n*-3 PUFA in sardine oil accounted for more than 81 % of the PUFA fractions in E_1 and E_6 with greater levels of EPA and DHA. However, the predominant PUFA found in the extraction processes was EPA, which was maximum in E_1 (17.1 %) followed by E_6 (15.5 %), E_3 (13.1 %), E_5 (12.9 %), E_4 (12.8 %), and E_2 (10.6 %). A maximum amount of DHA was observed in E_6 (5 %) followed by E_1 (4.2 %), E_2 (4 %), E_4 (3.8 %), E_3 (3.7 %), and E_5 (3.3 %), in descending order. No significant differences were noticed in the EPA and DHA contents of E_1 and E_6 ($p > 0.05$). An increase in linoleic acid (18:2n-6) and eicosadienoic acid (20:2n-6) was apparent in E_6 with lesser amount of *trans* fatty acids (0.04 %) as compared to $E_{1\text{-}5}$ ($\geq$ 0. 07 %) (Table 4.3). No significant difference was obtained between the EPA, DHA and total PUFA composition of E_6 compared to the conventional Bligh and Dyer method ($p > 0.05$). This led us to infer that the method followed by cooking and pressing was an efficient methodology for obtaining crude oil from the oil sardines without compromising the required nutritional values. Furthermore, because of the intrinsic difficulties in the phase separation and the high number of manipulations used in conventional extractions, E_6 may be advised as a convenient automated method for lipid extraction from fish samples. The methodology of solvent extractions and their distillation as in E_1-E_5 could potentially cause degradation or decomposition of thermally labile compounds, which results in serious health hazards. Moreover, E_6 has the advantages of requiring less laboratory material and time as well as absence of solvents.

Table 4.3 Fatty acid composition of the crude sardine oil obtained by different extraction modes

	E_1	E_2	E_3	E_4	E_5	E_6
Saturated						
14:0	8.6±0.86^a	12.9±1.27^{ab}	15.2±1.52^b	15.25±1.53^b	14.7±1.47^b	13.02±1.3^{ab}
15:0	0.9±0.09^a	0.72±0.07^a	0.7±0.07^a	0.72±0.07^a	0.75±0.08^a	0.7±0.07^a
16:0	14.2±1.43^a	21.68±2.18^b	20.07±2.01^b	19.59±1.95^b	22.26±2.22^b	21.65±2.17^b
17:0	0.39±0.04^a	0.49±0.05^a	0.52±0.05^a	0.71±0.07^a	0.93±0.09^a	0.63±0.06^a
18:0	5.07±0.51^a	4.73±0.47^a	3.76±0.38^a	3.84±0.38^a	4.05±0.41^a	4.23±0.41^a
20:0	0.45±0.05^a	0.36±0.04^a	0.26±0.03^a	0.28±0.03^a	0.25±0.03^a	0.34±0.03^a
22:0	0.11±0.01^a	0.25±0.03^a	0.94±0.09^a	0.3±0.03^a	0.11±0.01^a	0.39±0.04^a
24:0	0.9±0.09^a	0.22±0.02^a	0.04±0^b	0.1±0.01^a	0.03±0^b	0.07±0.01^{ab}
∑SFA	30.62±3.08^a	41.35±2.13^b	41.49±2.15^b	40.79±1.07^b	43.08±0.31^b	41.03±4.09^b
Monounsaturated						
14:1*n*-7	0.1±0.01^a	0.07±0.01^a	0.08±0.01^a	0.15±0.02^a	0.15±0.02^a	0.08±0.01^a
15:1*n*-7	ND	0.01±0^a	0.13±0.01^a	0.13±0.01^a	0.07±0.01^a	0.15±0.02^a
16:1*n*-7 *trans*	0.02±0^a	0.43±0.04^a	0.05±0.01^a	0.04±0^a	0.07±0.01^a	0.01±0^a
16:1*n*-7	5.9±0.59^a	13.24±1.32^b	15.04±1.5^b	15.6±1.55^b	14.68±1.47^b	13.04±1.3^b
18:1*n*-9 *trans*	0.02±0^a	0.07±0.01^a	0.01±0^a	0.06±0.01^a	0.01±0^a	0.02±0^a
18:1*n*-9	19.7±1.97^a	8.76±0.88^b	12.48±1.25^{ab}	9.04±0.9^b	12.36±1.24^{ab}	12.05±1.2^{ab}
20:1*n*-9	0.11±0.01^a	0.57±0.06^a	0.53±0.05^a	0.46±0.05^a	0.37±0.04^a	0.52±0.05^a
22:1*n*-9	ND	2.31±0.22^a	2.51±0.24^a	2.5±0.25^a	2.51±0.25^a	3.08±0.31^a
24:1*n*-9	0.5±0.05^a	0.75±0.08^a	0.26±0.03^a	0.23±0.02^a	0.1±0.01^a	0.24±0.02^a
∑MUFA	26.35±2.63^a	26.21±2.62^a	31.09±3.1^a	28.21±2.81^a	30.32±3.05^a	29.19±2.91^a
Polyunsaturated						
18:2*n*-6 *trans*	0.03±0^a	0.19±0.02^a	0.04±0^a	0.05±0.01^a	0.04±0^a	0.01±0^a
18:2*n*-6	1.24±0.12^a	1.65±0.17^a	1.92±0.19^a	1.89±0.19^a	1.89±0.19^a	1.99±0.2^a
18:3*n*-6	1.92±0.19^a	0.58±0.06^a	0.44±0.04^a	0.4±0.04^a	0.48±0.05^a	0.47±0.05^a
18:3*n*-3	1.87±0.18^a	0.18±0.02^a	0.26±0.03^a	0.39±0.04^a	0.26±0.03^a	0.29±0.03^a
20:2*n*-6	0.06±0.01^a	1±0.1^b	1.22±0.11^b	1.19±0.12^b	1.11±0.1^b	1.29±0.13^b
20:3*n*-6	0.33±0.03^a	0.34±0.03^a	0.3±0.03^a	0.07±0.01^b	0.27±0.03^a	0.07±0.01^b
20:4*n*-6	2.09±0.21^a	0.51±0.05^a	0.41±0.04^a	0.44±0.04^a	0.38±0.04^a	0.54±0.05^a
20:5*n*-3 EPA	17.1±1.7^a	10.59±1.05^b	13.06±1.3^{ab}	12.77±1.29^b	12.95±1.3^b	15.48±1.54^a
22:5*n*-3	1.92±0.19^a	0.22±0.02^b	0.48±0.05^b	0.27±0.03^b	0.34±0.03^b	1.37±0.14^a
22:6*n*-3 DHA	4.22±0.41^a	3.98±0.4^a	3.67±0.37^a	3.83±0.38^a	3.31±0.33^a	4.97±0.5^a
∑PUFA	30.78±1.04^a	19.24±1.92^b	21.8±2.16^{bc}	21.3±1.15^{bc}	21.03±1.1^{bc}	26.48±0.65^{ac}
EPA+DHA	21.32±2.11^a	14.57±1.45^b	16.73±1.67^b	16.6±1.67^b	16.26±1.63^b	20.45±2.04^a
∑*n*-3PUFA	25.11±0.48^a	14.97±1.49^b	17.47±1.75^b	17.26±1.74^b	16.86±1.69^b	22.11±2.21^a
∑n-6PUFA	5.64±0.56^a	4.08±0.41^a	4.29±0.41^a	3.99±0.4^a	4.13±0.41^a	4.36±0.44^a
∑*n*-3/∑*n*-6	4.45±0.43^a	3.67±0.63^a	4.07±0.27^a	4.33±0.35^a	4.08±0.12^a	5.07±0.02^a
∑*n*-6/∑*n*-3	0.22±0.003^a	0.27±0.008^a	0.25±0.23^a	0.23±0.03^a	0.24±0.04^a	0.2±0.02^a
∑PUFA/∑SFA	1.01±0.99^a	0.47±0.46^a	0.53±0.02^a	0.52±0.03^a	0.49±0.49^a	0.65±0.65^a
∑TRANS	0.07±0^a	0.69±0.07^a	0.1±0.01^a	0.15±0.02^a	0.12±0.01^a	0.04±0^a

E_1 – extraction using $CHCl_3$:MeOH (2:1); E_2 – Aqueous EtOH (40%); E_3 - EtOH (95%); E_4 - H_2O; E_5 Cooking/$CHCl_3$:MeOH (2:1); E_6 – Cooking/pressing. Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. Means with different superscripts (a, b etc) in the same row indicates a statistical difference ($p<0.05$). ND: not detected.

4.2.1.4. Spectral Analyses of Crude Sardine Oil (E_6)

The GC-MS of E_6 fatty acid methyl esters was determined to primarily study the chain length and the degree of unsaturation with the molecular ion (M^+). The following are the mass spectrometric data of the FAME derivatives (Fig 4.5).

Methyl Palmitate. EI-MS m/z (relative intensity, %): 270 (M^+, 61.11), 239 (15.74), 227 (31.48), 213 (7.41), 199 (14.81), 185 (12.96), 171 (12.96), 157 (7.41), 143 (31.48), 129 (11.11), 87 (74.07), 74 (100), 55 (18.52).

Methyl Oleate. EI-MS m/z (relative intensity, %): 296 (M^+, 20.00), 111 (76.67), 264 (33.33), 222 (26.67), 180 (18.33), 166 (23.33), 152 (23.33), 123 (23.33), 110 (38.33), 97 (75.00), 83 (70.00), 74 (66.67), 69 (78.33), 55 (100).

Methyl Linoleate. EI-MS m/z (relative intensity, %): 294 (M^+, 52.46), 263 (24.59), 220 (8.20), 178 (13.11), 164 (19.67), 150 (21.31), 136 (18.03), 123 (18.85), 109 (37.70), 95 (70.49), 81 (100), 67 (91.80), 55 (50.82).

Methyl Linolenate. EI-MS m/z (relative intensity, %): 292 (M^+, 16.67), 261 (5.00), 236 (6.67), 173 (6.67), 163 (6.67), 149 (20.00), 135 (20.00), 121 (25.00), 108 (56.67), 95 (58.33), 79 (100), 67 (56.67), 55 (35.00).

Methyl Arachidonate. EI-MS m/z (relative intensity, %): 318 (M^+, 1.82), 290 (1.82), 264 (1.82), 175 (5.45), 150 (7.27), 133 (7.27), 105 (30.91), 91 (70.91), 79 (100), 67 (80.00), 55 (49.09).

Methyl Eicosapentaenoate. EI-MS m/z (relative intensity, %): 315 (M^+, 1.67), 175 (6.67), 161 (8.33), 145 (11.67), 131 (18.33), 119 (31.67), 108 (31.67), 91 (70.00), 79 (100), 67 (68.33), 55 (48.33).

Methyl Docosahexaenoate. EI-MS m/z (relative intensity, %): 342 (M^+, 0.60), 145 (4.20), 131 (6.60), 119 (10.80), 108 (11.40), 91 (28.20), 79 (100), 67 (20.40).

The following are the mass spectrometric data of N-acyl pyrrolidide derivatives (Fig 4.5).

1-(Pyrrolidin-1-yl) hexadecan-1-one/Palmitoylpyrrolidine. EI-MS m/z (relative intensity, %): 309 (M^+, 16.00), 294 (2.00), 168 (8.00), 140 (10.00), 126

(16.00), 113 (100), 98 (8.00), 70 (12.00), 55 (14.00). 1-(Pyrrolidin-1-yl) octadec-9-en-1-one. EI-MS m/z (relative intensity, %): 335 (M+, 27.56), 250 (8.62), 236 (10.34), 208 (6.90), 196 (5.17), 182 (12.07), 126 (53.45), 113 (100), 98 (18.97), 85 (8.62), 72 (20.69), 55 (27.59).

1-(Pyrrolidin-1-yl) octadeca-9,12-dien-1-one. EI-MS m/z (relative intensity, %): 333 (M^+, 77.97), 290 (10.17), 236 (15.25), 222 (20.34), 182 (16.95), 168 (15.25), 140 (22.03), 126 (44.07), 113 (100), 98 (25.42), 70 (42.37), 55 (49.15).

1-(Pyrrolidin-1-yl) octadeca-9,12,15-trien-1-one. EI-MS m/z (relative intensity, %): 331 (M^+, 44.00), 182 (22.00), 168 (24.00), 140 (26.00), 126 (60.00), 113 (100), 98 (30.00), 72 (64.00), 55 (42.00).

1-(Pyrrolidin-1-yl) icosa-5,8,11,14-tetraen-1-one. EI-MS m/z (relative intensity, %): 357 (M^+, 18.97), 232 (10.34), 180 (10.34), 126 (13.79), 113 (100), 85 (17.24), 70 (22.41), 55 (27.59).

1-(Pyrrolidin-1-yl) icosa-5,8,11,14,17-pentaen-1-one. EI-MS m/z (relative intensity, %): 355 (M^+, 3.85), 286 (7.69), 232 (7.69), 126 (13.46), 113 (100), 85 (17.31), 72 (26.92), 55 (21.15).

1-(Pyrrolidin-1-yl) octadeca-9, 12-dien-1-one. EI-MS m/z (relative intensity, %): 381 (M^+, 3.91), 312 (7.05), 272 (7.29), 232 (16.22), 218 (15.76), 192 (8.24), 166 (23.67), 153 (22.85), 113 (100), 98 (46.62), 72 (21.98).

Mass spectrometry of FAME with EI ionization provides meager information only related to the structure of fatty acids, particularly for LC-PUFAs. The molecular ions for SFA and MUFA FAME were conspicuous as obvious from the mass spectra, and hence the molecular weight is discernible from the EI-MS spectra of FAME. An ion at $(M - 31)^+$ represents the loss of the $-OCH_3$ group, thus confirming the molecular structure as methyl ester. In the mass fragmentation pattern of FAME, the base peak was assigned to be the 1-methoxyethenol moiety (m/z = 74) obtained by McLafferty rearrangement per se with the loss of the McLafferty ion (m/z = 222). The molecular ion undergoes further rearrangement to furnish 1-methoxyprop-2-en-1-ol (m/z = 88). The characteristic ion for SFA is formation of the McLafferty ion

(m/z = 74) by McLafferty rearrangement, while MUFA methyl esters exhibited the formation of a stable and abundant ion at M - 74 after the loss of the McLafferty ion. The spectra further contained lower m/z fragment ions at a difference of m/z = 14, definitely supporting the general structure of FAME, viz., $[-CH_3OCO-(CH_2)_nCH_3]^+$ in the homologous series. The McLafferty ion was found to be less conspicuous in EI-MS of LC-PUFAs having more double bonds (n > 4), which undergoes complex rearrangement under high-energy EI conditions. The abundance of these fragment ions in SFA and MUFA-FAME was found to be higher than in the EI-MS spectra of LC-PUFAs. Formation of the cyclic tropylium ion (m/z = 91) in fatty acids with four or more double bonds was apparent in PUFAs. Cleavage of the tail from the *n*-6 terminal moiety in the methylene-interrupted bond to yield a fragment ion at m/z = 150 was found to be characteristic of *n*-6 PUFA methyl esters, while for *n*3 PUFA methyl esters the characteristic fragment ion was apparent at m/z = 108 following cleavage of the tail from the *n*-6 terminal moiety. The EI-MS spectrum of methyl linoleate has an abundant molecular ion (m/z = 294) and base peak as the McLafferty ion (m/z = 74). In methyl linolenate, a fragment ion at m/z = 150 was obvious, which is characteristic for fatty acids with an *n*-6 terminal moiety, while one at m/z = 108 defines an *n*3 terminal group as in methyl eicosapentaenoate and methyl docosahexaenoate. In the mass fragmentation pattern of methyl arachidonate the molecular ion is barely distinguishable, but the characteristic ion for the *n*3 moiety, at m/z = 108, is apparent. In the mass fragmentation pattern of LC-PUFAs with more double bonds ($n \geq 4$), the tropylium ion (m/z = 91) does stand out as the diagnostic fragment ion. However, in FAME EI-MS spectra it is difficult to determine the location of the double bond from the spectra. The reason for this is the migration of the double bond along the long aliphatic chain due to destabilization of the positive charge by electron ionization in the mass spectrometer. This drawback is encountered by the EI-MS of fatty acid pyrrolidine derivatives (N-acyl pyrrolidides), which minimize the migration, as they are able to stabilize the charge on the heterocyclic pyrrolidine moiety. In the mass fragmentation pattern of N-acyl pyrrolidides, the base peak was assigned to be the McLafferty rearrangement ion, 1-(pyrrolidin- 1-yl) ethanol (m/z = 113), with the concurrent loss of the M - 113

fragment ion. The molecular ion undergoes further rearrangement to furnish 1-(pyrrolidin-1-yl) prop-2-en-1-ol (m/z = 127). A uniform distribution of fragment peaks is apparent at every m/z = 14 units, except in the vicinity of the double bond, where the interval is m/z = 12 units. For example, in the mass spectrum of 1-(pyrrolidin-1-yl) octadeca-9,12,15-trien-1-one, the double bonds in positions 9 and 12 are located by the gaps of m/z = 12 units between ions at m/z = 196, 208 and 236, 248, respectively.

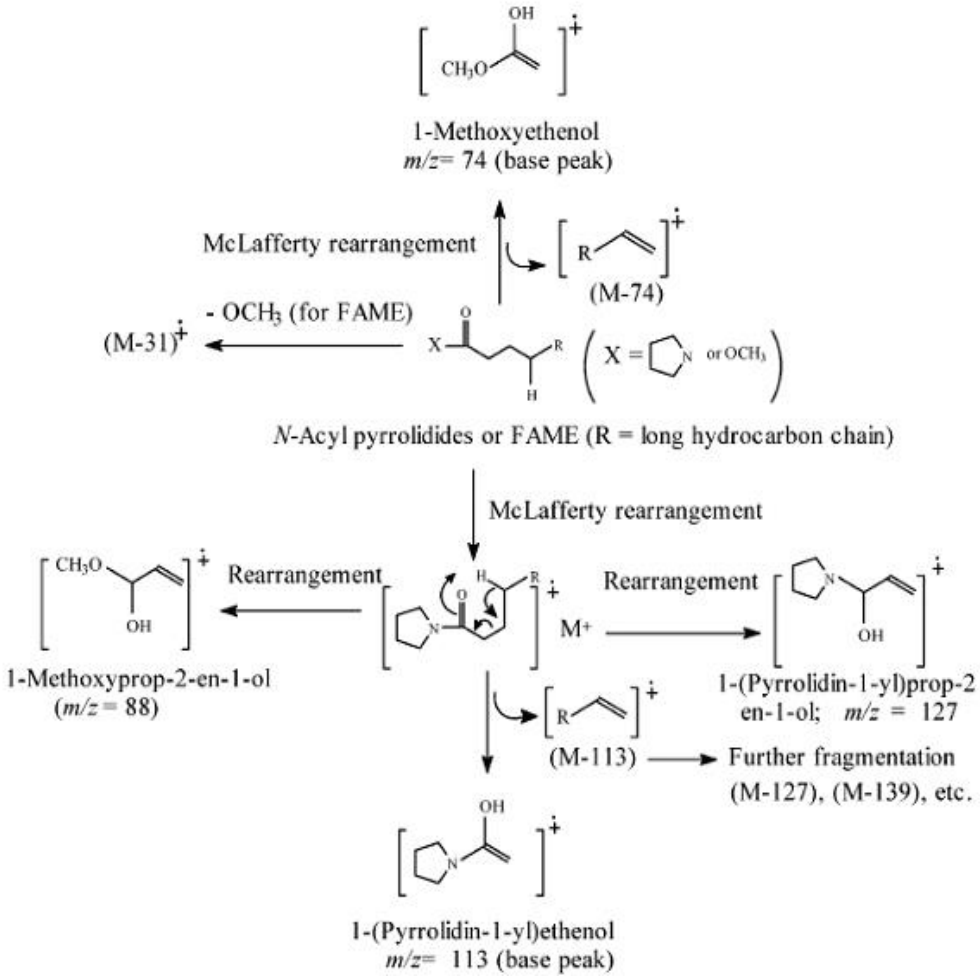


Fig.4.5. Tentative mass fragmentation pattern of FAME and N-acyl pyrrolidides

The oil obtained by extraction process with cooking and pressing (E_6) was selected as the optimum method by evaluating the quality markers of the crude oil, and was further characterized by spectral analysis as follows.

^{1}H-NMR analysis

The signature peaks and relative abundance of different fatty acids of E_6 were identified from the ^{1}H-NMR spectral features (Fig. 4.6A). Peak assignments were performed with the aid of literature data (Siddiqui *et al*., 2003; Vidal *et al*., 2012), and by recording spectra of standard compounds. The signals at δ 0.85 - 0.92 (Fig. 4.6A(a)), was due to the overlapping of the triplet of methylic protons of saturated, monounsaturated *n*-9 and/or *n*-7 acyl groups centered at δ 0.88 and of the triplet of methylic protons of unsaturated *n*-6 acyl groups centered at δ 0.889. The presence of unsaturated *n*-3 lipids is further confirmed by a sharp triplet at δ 0.974, a structural feature for methyl proton (-C**H**$_3$) in *n*-3 PUFA (Fig. 4.6A(b). The ratio of this peak area (δ 0.969) to that of overall peak area of methyl group resonances (broad triplet at δ 0.878 + sharp triplet at δ 0.969) enabled to establish the relative abundance of *n*-3 PUFA (26.5 %), which were comparable to that obtained by GC analysis (Table 4.3). The signals at δ 1.18 – 1.43 can be explained due to the methylene envelope (–(C**H**$_2$)$_n$) in the long alkyl chain (Fig. 4.6A(c)). This region has two main peaks, one at δ 1.257 corresponding to the methyl protons of saturated acyl groups and the other one at δ 1.300 associated with the overlapping of methylenic protons of the several unsaturated acyl groups. The signal at δ 1.52 - 1.66, is due to methylenic protons in β-position in relation to the >C=O group (-OCO-CH$_2$-C**H**$_2$-), except those of DHA, EPA and arachidonic acyl groups (Fig. 4.6A(d)). The signals at δ 1.66–1.75 was assigned due to the methylenic protons in the β-position in relation to the >C=O group (-OCO-CH$_2$-C**H**$_2$-) of EPA and arachidonic acyl groups (Fig. 4.6A(e)). The peaks at δ 1.92 - 2.15 indicated the –C**H**$_2$ group except for DHA acyl group in β position in relation to >C=O group (Fig. 4.6A(f)). The peaks at δ 2.25-2.36 indicated –OCO-C**H**$_2$- *n*-3 fatty acid protons except DHA acyl group (Fig. 4.6A(g)). The peaks at δ 2.36 - 2.42 indicated –OCO-C**H**$_2$- *n*-3 fatty acid protons of DHA acyl group (Fig. 4.6A(h)). The sandwiched CH$_2$ *bis*-allylic protons (-CH=CH-C**H**$_2$-CH=CH-) were overlapped and displayed at δ 2.74 – 2.92 (multiplets) (Fig. 4.6B(a)). These *bis*-allylic peaks indicate the predominance of PUFA in E_6. The two double quadruplets at δ 4.95 – 5.1 could be assigned to the protons of the carbon in position 1 (-C**H**=CH$_2$) of *n*-1 acyl groups (Fig. 4.6B(c)). The signals at δ 5.22 – 5.3 ppm indicate the *sn*-2 methine proton (-C**H**OCOR) of glyceride portion (Fig. 4.6B(d)). This

methine proton is sandwiched by two α-(CH_2OCO) moieties, thereby highly deshielded. The characteristic signals of olefinic protons (-C**H**=C**H**-) appeared at δ 5.31-5.64 ppm (Fig. 4.6B(e)). ^{1}H-NMR spectrum displayed a multiplet in the region δ 5.75 – 5.87 ppm which can be assigned to the protons of the carbon in position-2 (-C**H**=CH-) of *n*-1 acyl groups (Fig. 4.6B(f)). The mean unsaturation index (UI) was estimated by the ratio of the integral area of the multiplet at δ 5.366 (olefinic protons) with respect to those of the triplets at δ 0.878 and δ 0.969 (all the terminal methyl groups) (Fiori *et al*., 2012). The value for UI for E_6 (1.39 obtained by ^{1}HNMR analysis) was in good agreement with those obtained by GC analysis {1.54 calculated by UI = [(% MUFA x 1) + (% dienoic x 2) + (% trienoic x 3) + (% tetraenoic x 4) + (% pentaenoic x 5) + (% hexaenoic x 6)]/100} when taking into consideration the relative errors of measurement of both methodologies. Finally, the secondary products deriving from lipid oxidation (such as peroxides) due to possible damage during extraction were not detectable in the ^{1}H-NMR spectra of E_6, which correlate with the quality parameter analyses as described earlier. These observations have been further validated with the ^{13}C-NMR/$DEPT_{135}$ and infra-red analyses data of the crude sardine oil as follows.

^{13}C and $DEPT_{135}$ NMR analysis

The structural attributions of E_6 were also confirmed by the analysis of the corresponding ^{13}C and $DEPT_{135}$ NMR spectra (Fig. 4.6C and D). The carbonyl region of oil points out the utility of ^{13}C NMR while studying the acyl stereo-specific positions of fatty acids. In the carbonyl region (δ 180-172), the peaks at 172-178 were attributed to >**C**=O group present in the crude sardine oil (Fig. 4.6C(a)). The peaks at δ 177.477, 173.286 and 173.254 were characteristics to the carbonyl group of the fatty acid chain esterified at *sn*-1, 3 glycerol positions in the lipid. The low frequency set (δ 173.019 & 172.873) in the carbonyl region includes the *sn*-2 glycerol position chains. The signals from >**C**=O of *sn*-1, 3 chains are shifted at greater frequency from those of carbonyls of *sn*-2 chains. This shift difference, which is consistently detected for all chains, was explained by noting that >C=O groups of *sn*-2 position chains experience two *γ –gauche* interactions against just one

interaction intervening for carbonyls of *sn*-1, 3 chains (Howarth *et al*., 1995). Thus ^{13}C-NMR spectroscopy has enabled the analysis of the positional distribution of fatty acids in the crude TAGs of oil sardines.

The olefinic region (δ 134-124) region gave rise to signals from various double bonds present in the fatty acids of the crude oil (Fig. 4.6C(b)). In this region, intense signals were observed between δ 132.0 and 127.0, which were attributed to the *n*-3 **C** atoms in polyunsaturated fatty acids. The greater concentration of *n*-3 fatty acids in E_6 (22.1 % of total fatty acids) was reflected in the greater intensity signals at δ 129.72 and 130.456 that were assigned to the two olefinic n-3 carbons (Aursand *et al*., 1993; Siddiqui *et al*., 2003). Due to the greater concentration of EPA, which contain five non-conjugated double bounds, the majority of the high intensity olefinic resonances were observed at δ 127.8 – 129.1 ppm, which was originated from this fatty acid. Resonances from other unsaturated fatty acids were also found in this region, and the resonance at δ 128.796 was assigned to **C5** and **C6** carbons of 20:5*n*-3 (Aursand *et al*., 1993). The MUFAs were assigned in δ 129.720-130.008 region. The intensity of the resonances at δ 129.720 and 129.931 showed the presence of C8 and C10 carbons of 18:1 in E_6.

The glycerol carbons of mono-, di- and triacylglycerols, resonated in the spectral region from δ 60-72 (Fig. 4.6C(c)). The resonances from the **C**H signals of *sn*-2 carbons and *sn*-1, 3 positions (Aursand & Grasdalen 1992) appeared at δ 68.969 and 62.143, respectively. This was further confirmed by the $DEPT_{135}$ analysis. It was apparent from the ^{13}C-NMR that the resonances of the glycerol *sn*-2 shifted to greater frequencies from those of the *sn*-1, 3 carbons.

The up-field aliphatic region of the spectra (δ 35 - 10) displayed a greater level of *n*-3 (-**C**H_3 δ 14.271) fatty acids and lesser levels of *n*-6 (-**C**H_2 δ 31.542) fatty acids in E_6 (Fig. 4.6C(d)). The presence of the –CH_2 groups were further confirmed by the $DEPT_{135}$ analysis (Fig. 4.6D). This was in compliance with the $n-3/n-6$ ratios and the contribution of $n-3$ fatty acids in the samples found by GLC.

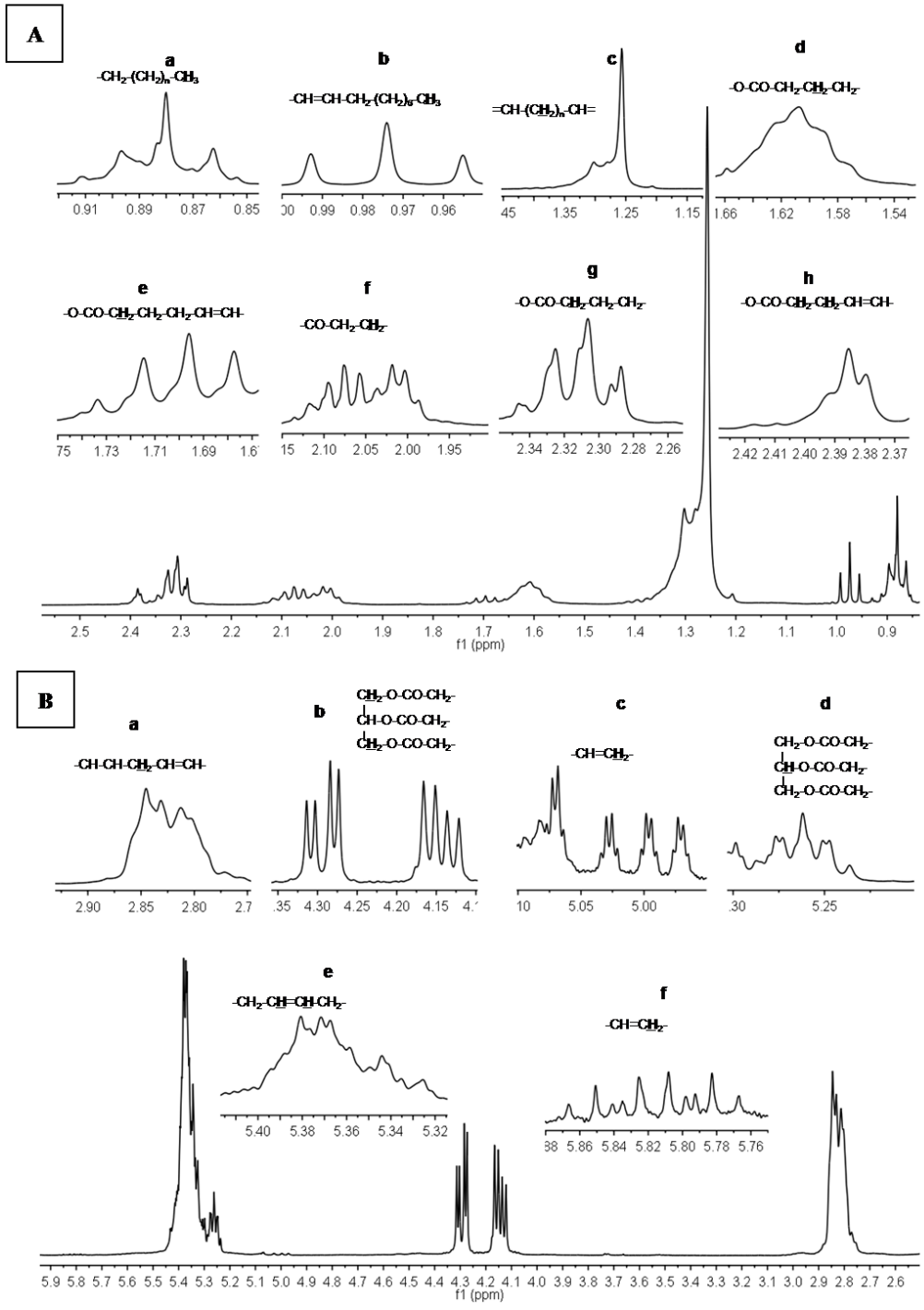


Fig. 4.6. (**A**) ^{1}H-NMR spectrum (δ 0 – 2.5 ppm) region of the oil obtained by cooking followed by pressing method (E_6). The insets represent the ^{1}H-NMR region at (a) δ 0.85 – 0.92 ppm, (b) δ 0.92 – 1.0 ppm, (c) δ 1.18 – 1.43 ppm, (d) δ 1.52 – 1.66 ppm, (e) δ 1.66 – 1.75 ppm, (f) δ 1.92 – 2.15 ppm, (g) δ 2.25 – 2.36 ppm and (h) δ 2.36 – 2.42 ppm; (**B**) (a) δ 2.74 – 2.92 ppm, (b) δ 4.12 – 4.34 ppm, (c) δ 4.95 – 5.1 ppm, (d) δ 5.22 – 5.3 ppm, (e) δ 5.31 – 5.64 ppm, and (f) δ 5.75 – 5.87 ppm.

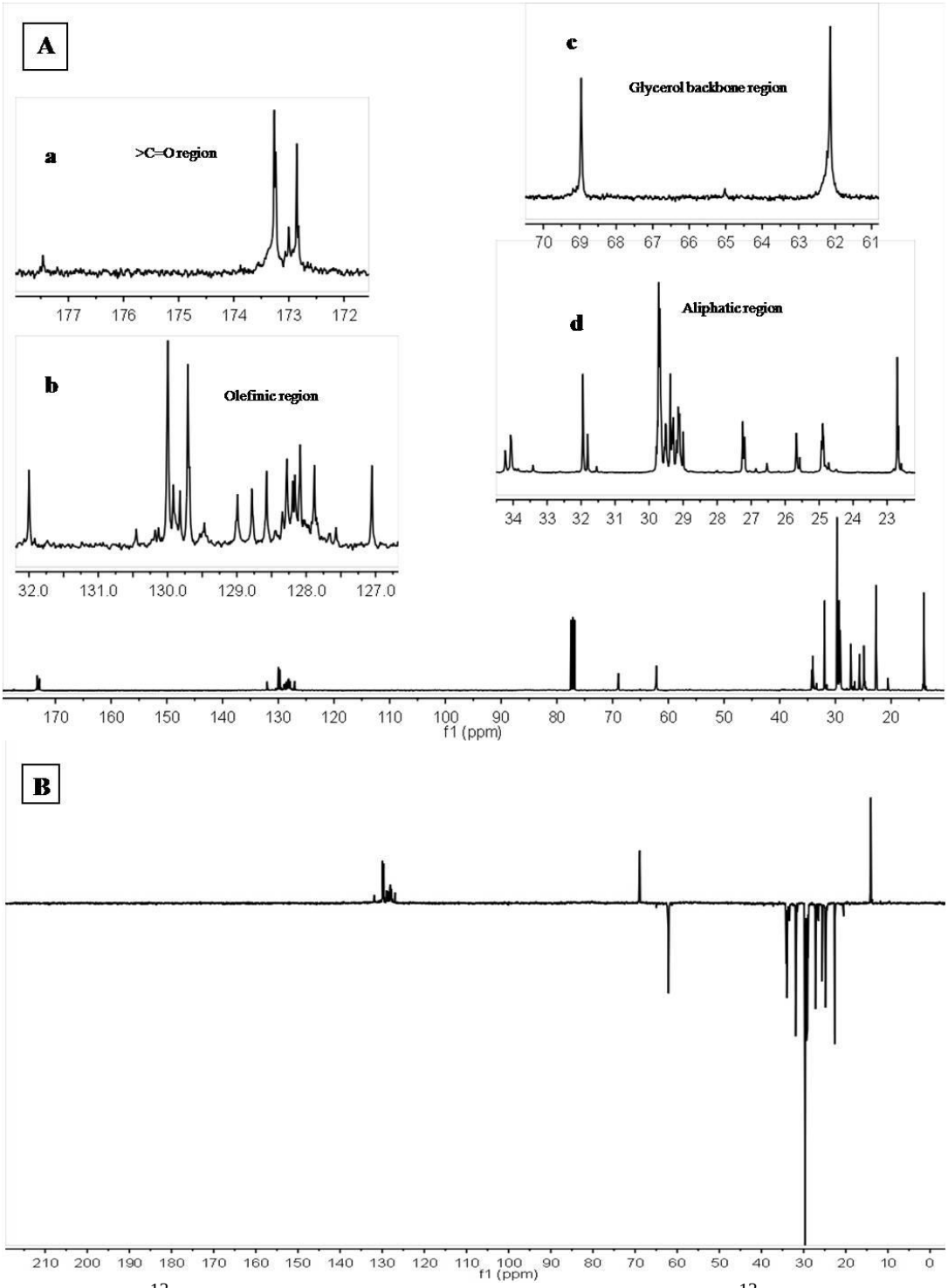


Fig. 4.6 (**C**) ^{13}C-NMR spectrum of E_6. The insets represent the ^{13}C-NMR region at (**a**) δ 180 - 172 ppm, (**b**) δ 134 - 124 ppm, (**c**) δ 60 - 72 ppm and (**d**) δ 35 - 10 ppm. (**D**) The $DEPT_{135}$ - NMR spectra of oil obtained using cooking and pressing method (E_6).

FTIR analysis

FTIR spectrum of the crude oil has a weak band (a) associated with the overtone of the glyceride ester carbonyl absorption at 3471.42 cm^{-1} (Fig. 4.7). The dominant spectral features in the region from 3050-2800 cm^{-1} were the =C-H stretching vibrations. The stretching vibration of the *cis*-olefinic double bonds was found at 3012.01 cm^{-1} (b) and the higher frequency of this band indicated its richness in polyunsaturated acyl groups. The two bands (c and d) resulting from the methylene asymmetrical and symmetrical stretching vibrations appear at 2925.17 and 2853.39 cm^{-1}, respectively. The ratio of absorbances of bands b and d was taken as a measure of the degree of unsaturation of the oil. This ratio was found to be > 0.50 for E_6 thereby displaying the higher degree of unsaturation of this oil in relation to common edible oils such as olive (Ac/Af = 0.17, where A is absorbance), rapeseed (Ac/Af = 0.23), and corn (Ac/Af = 0.27) oils (Guillen & Cabo 1997). Band e, resulting from the stretching vibration of the >C=O group of triglyceride, appeared at 1746.90 cm^{-1}. The small band (f) assigned was due to the disubstituted *cis* C=C of the unsaturated acyl groups, appeared at 1654.59 cm^{-1}. Band g, resulted from the bending vibrations of the CH_2 and CH_3 aliphatic groups, appeared at 1463.28 cm^{-1}. The band h at 1376.58 cm^{-1}, resulted from symmetrical bending vibrations of the CH_3 groups, whereas band i (1238.26 cm^{-1}) was associated with the stretching vibration of the C–O ester groups. The band j at 1163.45 cm^{-1} was associated with the bending vibration of the -CH_2 group. The bands represented by k, l, and m depicted the fingerprint region.

4.2.2. Refining of Crude Sardine Oil

The crude sardine oil obtained by using the optimum extraction mode (E_6) had a brownish color with a fishy smell. In order to refine this oil, degumming, neutralization, bleaching and deodorization steps were sequentially performed to obtain refined sardine oil (RO).

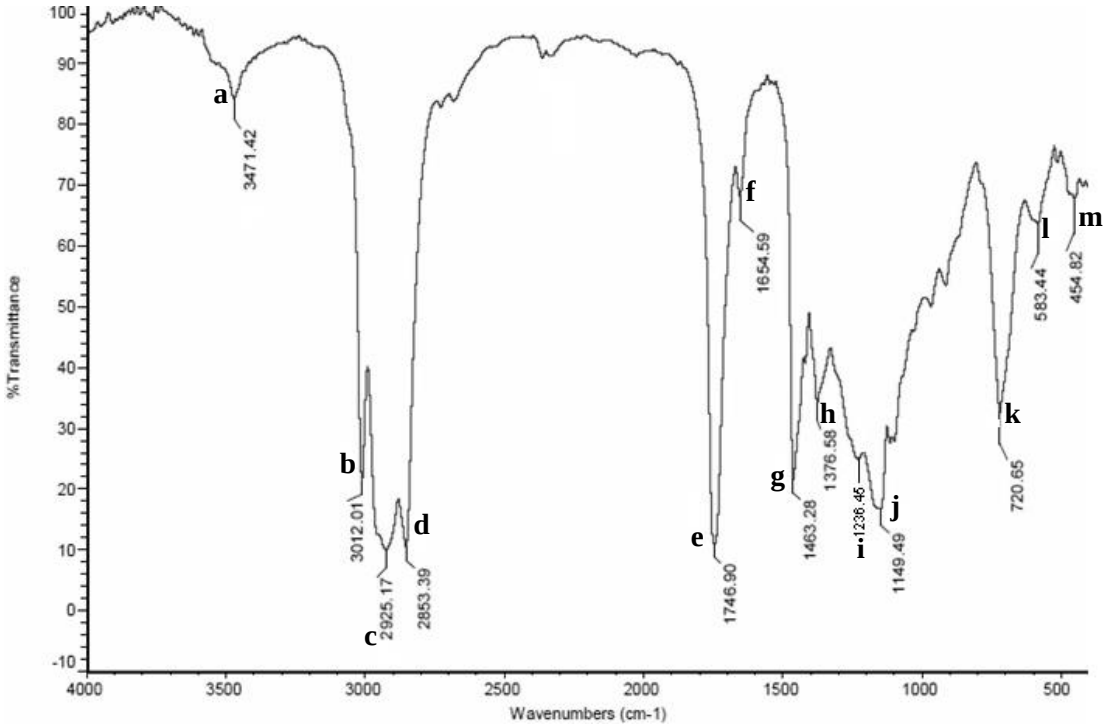


Fig. 4.7. FTIR spectrum of oil obtained using cooking and pressing method (E_6).

4.2.2.1. Degumming and Neutralization of Crude Sardine Oil

4.2.2.1A.Yield, P.V., pA.V., TOTOX, TBARS Values and Lovibond Color of the Sardine Oil after Degumming and Neutralization

Degumming with phosphoric acid (DG_1) yielded a dark residue (14 %, w/w) with 86 % oil recovery (w/w), whereas oxalic acid treatment (DG_3) yielded a dark residue (9.8 %, w/w) with 90.2 % oil recovery (Table 4.4). An increase in FFA (5.6 – 7.8 %) was observed for oil after degumming in relation to E_6 (4.4 %) (Table 4.4), which was expected due to the trace amount of acids added. P.V exhibited maximum reduction for DG_1 followed by DG_3, DG_2 and DG_4. DG_{1-4} observed lesser p.A.V in relation to E_6 with maximum % reduction observed for DG_1 (16 %) TBARS value showed 19.4 % reduction for DG_1 in relation to E_6. Initial L*, a*, b* values of E_6 were recorded to be 50.25, 7.58, and 42.51, respectively, which indicated the brownish orange color of the oil due to the deposition of dietary carotenoids (Luterotti *et al*., 1999). An increase in L*, a* (51.02 and 8.0, respectively) and a reduction in b* value (42.0) were observed for DG_1 whereas other degumming modes showed no substantial changes in the L*, a*, b* values (Table 4.4). This increase in L^* and a^* values could

be attributed to the increase in free fatty acids (FFA) content, resulting in formation of lipid–protein complexes, thereby consequently increasing color (Crexi *et al*., 2011).

The neutralization of DG_1 afforded a dark residue (6.6 g) with 80.4 % oil yield (w/w) with respect to CSO. The neutralization step effectively reduced the FFA value of DG_1 to 3.21 % (Table 4.4). Similarly, a reduction in P.V., pA.V. and TOTOX values was observed after neutralization ($p > 0.05$) (Table 4.4). A reduction in the TBARS value of neutralised oil in relation to E_6 was approximately 35 %. Neutralized oil was lighter (L* = 51.34) and more yellowish (b* = 43.65) in color than E_6. No reduction in redness (a* = 8.21) indicated that neutralization process might not have significantly removed the carotenoids pigments (Table 4.4). Neutralization increased the chroma values and decreased the hue-angle values of the oil.

Table 4.4 Characteristics of the sardine oil after degumming and neutralization

	E_6	DG_1	DG_2	DG_3	DG_4	Neutralized Oil
Yield (%)		86	93.06	90.05	92.56	80.36(93.44)
FFA	4.4 ± 0.44^{ab}	7.85 ± 0.79^{a}	6.98 ± 0.71^{ab}	6.01 ± 0.6^{ab}	5.65 ± 0.57^{ab}	3.21 ± 0.32^{b}
P.V	11.88 ± 1.18^{a}	7.2 ± 0.71^{ab}	8.01 ± 0.8^{ab}	7.56 ± 0.76^{ab}	8.25 ± 0.83^{ab}	6.09 ± 0.61^{b}
pA.V	16.2 ± 1.62^{a}	13.6 ± 1.36^{ab}	14.1 ± 1.41^{ab}	14.68 ± 1.47^{ab}	15.01 ± 1.5^{a}	10.01 ± 1^{b}
TOTOX	39.96 ± 1.98^{a}	28 ± 2.78^{b}	30.12 ± 3.01^{b}	29.8 ± 2.99^{b}	31.51 ± 3.16^{b}	22.19 ± 0.22^{c}
TBARS	6.18 ± 0.62^{a}	4.98 ± 0.5^{a}	5.21 ± 0.52^{a}	5.69 ± 0.57^{a}	5.88 ± 0.59^{a}	4.02 ± 0.4^{a}
Lovibond Color						
L	50.25 ± 0.03^{a}	51.02 ± 1.11^{a}	49.58 ± 2.97^{a}	50.01 ± 1.01^{a}	50.21 ± 0.02^{a}	51.34 ± 2.13^{a}
a	7.58 ± 0.75^{a}	8 ± 0.8^{a}	7.45 ± 0.75^{a}	7.58 ± 0.76^{a}	7.88 ± 0.79^{a}	8.21 ± 0.82^{a}
b	42.51 ± 1.26^{a}	42.01 ± 0.2^{a}	43.01 ± 2.29^{a}	42 ± 4.2^{a}	42.01 ± 2.2^{a}	43.65 ± 1.37^{a}

DG_1 – phosphoric acid; DG_2 – glacial acetic acid; DG_3 - oxalic acid; DG_4 - citric acid;. Data are expressed as mean ± standard deviation of three replicates. Means with different superscripts (a, b etc) in the row indicates a statistical difference ($p<0.05$). Other notations are as Table 4.2.

4.2.2.1B. Fatty acid Composition after Degumming and Neutralization

The analysis of fatty acid profile of sardine oil after degumming or neutralization observed no significant difference ($p>0.05$) as compared to the control oil E_6 (Table 4.5). However, an insignificant reduction in EPA, ∑PUFA, and ∑*n*-3PUFA might be due to the involvement of heat during the process of degumming and neutralization. This was further evident by the formation of minor quantities of *trans* fatty acids, especially 16:1*n*-7 *trans* in the neutralized oil. The chromatogram after neutralization was shown in Fig. 4.8.

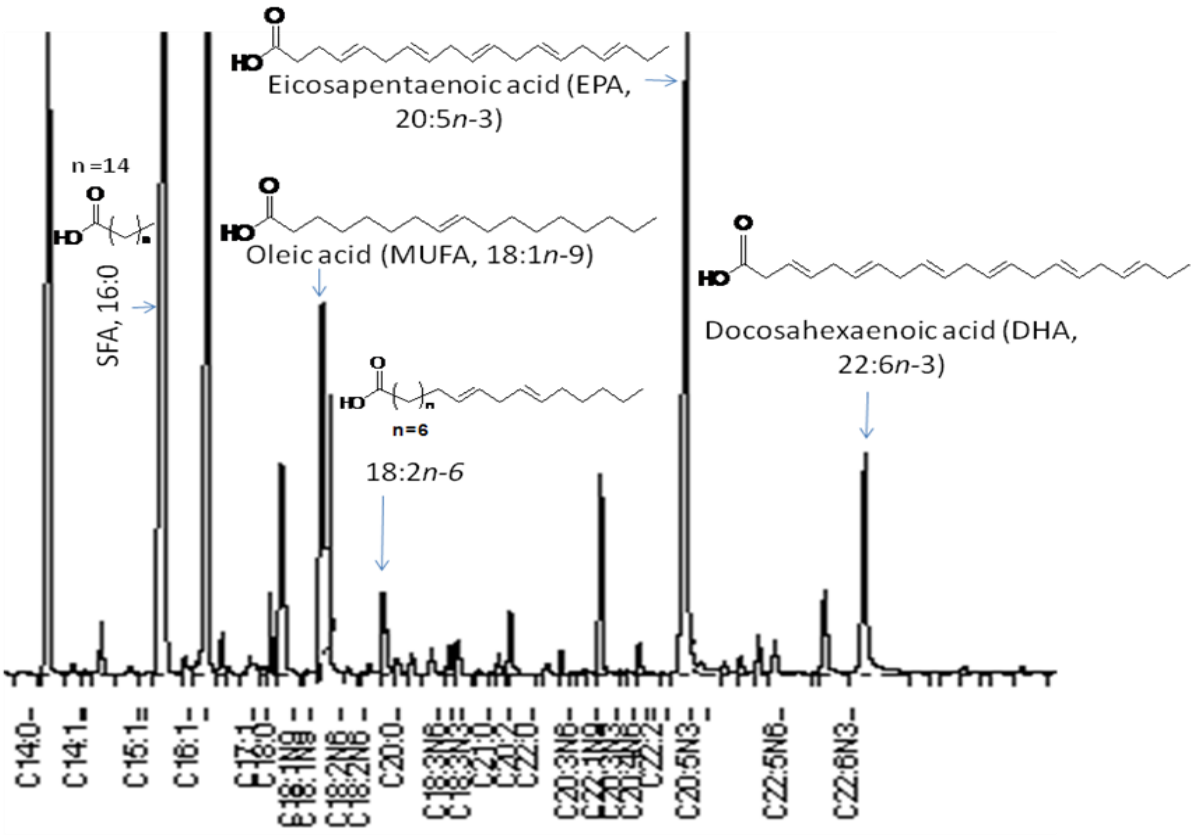


Fig.4.8. Fatty acid chromatogram of neutralized oil

Table 4.5 Fatty acid composition of the sardine oil after degumming and neutralization

	E_6	DG_1	DG_2	DG_3	DG_4	Neutralized Oil
Saturated						
14:0	13.02±1.3^a	13.01±1.3^a	13±1.2^a	12.86±1.29^a	13.11±1.31^a	12.28±1.23^a
15:0	0.7±0.07^a	0.68±0.07^a	0.69±0.07^a	0.71±0.07^a	0.7±0.07^a	0.76±0.08^a
16:0	21.65±1.18^a	20.65±0.07^a	21.02±2.1^a	21.41±3.14^a	20.89±0.08^a	20.94±2.08^a
17:0	0.63±0.06^a	0.68±0.07^a	0.69±0.07^a	0.64±0.06^a	0.64±0.06^a	0.69±0.07^a
18:0	4.23±0.42^a	4.34±0.43^a	4.36±0.44^a	4.37±0.44^a	4.32±0.43^a	4.81±0.48^a
20:0	0.34±0.03^a	0.35±0.04^a	0.36±0.04^a	0.35±0.04^a	0.34±0.03^a	0.39±0.04^a
22:0	0.39±0.04^a	0.38±0.04^a	0.37±0.04^a	0.39±0.04^a	0.38±0.04^a	0.29±0.03^a
24:0	0.07±0.01^a	0.06±0.01^a	0.07±0.01^a	0.06±0.01^a	0.03±0^a	0.03±0^a
∑SFA	41.03±1.11^a	40.15±1.03^a	40.56±2.97^a	40.79±0.09^a	40.41±1.02^a	40.18±2.01^a
Monounsaturated						
14:1*n*-7	0.08±0.01^a	0.08±0.01^a	0.07±0.01^a	0.06±0.01^a	0.01±0^a	0.07±0.01^a
15:1*n*-7	0.15±0.02^a	0.17±0.02^a	0.15±0.02^a	0.16±0.02^a	0.15±0.02^a	0.18±0.02^a
16:1*n*-7 *trans*	0.01±0^a	0.02±0^a	0.01±0^a	0.01±0^a	0.01±0^a	0.03±0^a
16:1*n*-7	13.04±1.3^a	13.21±1.32^a	13.35±1.34^a	13.31±1.32^a	13.41±1.34^a	13.41±1.65^a
18:1*n*-9 *trans*	0.02±0^a	0.01±0^a	0.01±0^a	0.01±0^a	0.01±0^a	0.03±0^a
18:1*n*-9	12.05±1.2^a	12.01±1.2^a	12±1.2^a	11.89±1.19^a	11.21±1.12^a	10.49±1.05^a
20:1*n*-9	0.52±0.05^a	0.53±0.05^a	0.54±0.05^a	0.51±0.05^a	0.62±0.06^a	0.61±0.06^a
22:1*n*-9	3.08±0.31^a	3.11±0.31^a	3.26±0.33^a	3.16±0.31^a	3.24±0.33^a	3.1±0.3^a
24:1*n*-9	0.24±0.02^a	0.36±0.04^a	0.31±0.03^a	0.41±0.04^a	0.26±0.03^a	1.05±0.11^a
∑MUFA	29.19±2.91^a	29.5±2.95^a	29.7±2.97^a	29.52±2.95^a	28.92±2.89^a	28.98±3.2^a
Polyunsaturated						
18:2*n*-6 *trans*	0.01±0^a	0.01±0^a	0.01±0^a	0.01±0^a	0.01±0^a	0.03±0^a
18:2*n*-6	1.99±0.2^a	1.81±0.18^a	1.82±0.18^a	1.85±0.19^a	1.86±0.18^a	1.89±0.19^a
18:3*n*-6	0.47±0.05^a	0.41±0.04^a	0.45±0.05^a	0.46±0.05^a	0.48±0.05^a	0.44±0.04^a
18:3*n*-3	0.29±0.03^a	0.7±0.07^a	0.71±0.07^a	0.31±0.03^a	0.32±0.03^a	0.71±0.07^a
20:2*n*-6	1.29±0.13^a	1.27±0.13^a	1.29±0.13^a	1.29±0.13^a	1.29±0.13^a	1.26±0.13^a
20:3*n*-6	0.07±0.01^a	0.12±0.01^a	0.08±0.01^a	0.09±0.01^a	0.09±0.01^a	0.13±0.01^a
20:4*n*-6	0.54±0.05^a	0.5±0.05^a	0.51±0.05^a	0.52±0.05^a	0.53±0.05^a	0.09±0.01^a
20:5*n*-3 EPA	15.48±1.56^a	15.08±1.51^a	15.31±1.52^a	15.41±1.54^a	15.24±1.52^a	15.02±1.5^a
22:5*n*-3	1.37±0.14^a	0.88±0.09^a	0.9±0.09^a	1.26±0.13^a	1.27±0.13^a	0.7±0.07^a
22:6*n*-3 DHA	4.97±0.5^a	5.26±0.53^a	5.31±0.53^a	5.11±0.51^a	5.12±0.51^a	5.28±0.53^a
∑PUFA	26.48±2.67^a	26.04±2.6^a	26.39±2.64^a	26.31±2.63^a	26.21±2.62^a	25.55±0.55^a
EPA+DHA	20.45±2.06^a	20.34±2.03^a	20.62±2.06^a	20.52±2.05^a	20.36±2.04^a	20.3±2.03^a
∑*n*-3PUFA	22.11±1.23^a	21.92±2.19^a	22.23±2.22^a	22.09±2.21^a	21.95±2.2^a	21.71±2.17^a
∑*n*-6PUFA	4.36±0.44^a	4.11±0.41^a	4.15±0.42^a	4.21±0.42^a	4.25±0.43^a	3.81±0.38^a
∑*n*-3/∑*n*-6	5.07±1.07^a	5.33±0.53^a	5.36±0.54^a	5.25±0.53^a	5.16±0.52^a	5.7±2.71^a
∑*n*-6/∑*n*-3	0.2±0.2^a	0.19±0.02^a	0.19±0.02^a	0.19±0.02^a	0.19±0.02^a	0.18±0.18^a
∑PUFA/∑SFA	0.65±0.65^a	0.65±0.07^a	0.65±0.07^a	0.65±0.07^a	0.65±0.07^a	0.64±0.64^a
∑TRANS	0.04±0^a	0.04±0^a	0.03±0^a	0.03±0^a	0.03±0^a	0.09±0^a

E_6 – Crude sardine oil, DG_1 – phosphoric acid; DG_2 – glacial acetic acid; DG_3 - oxalic acid; DG_4 - citric acid. All other notations are as in Table 4.3. Data are expressed as mean ± standard deviation of three replicates. Means with different superscripts (a, b etc) in the row indicates a statistical difference ($p<0.05$).

4.2.2.2. Bleaching of the Neutralized Sardine Oil

The effect of different adsorbents on the qualities of the neutralized oil (B_0) were studied under three factors, such as (1) adsorbents when added individually, (2) combinations of selected adsorbents and (3) effect of three different concentrations of activated charcoal:Fuller's earth (1%, 3% and 5%) and contact time (20, 40 & 60 min) on the oil. The results are discussed as follows.

4.2.2.2A. Recovery and Characteristics of Bleached Sardine Oil

4.2.2.2A'. Bleaching with Individual Adsorbents

Table 4.6 presents the experimental values of the recovery percent, free FFA, P.V, pA.V, TOTOX, TBARS, CIELAB color coordinates (L*, a*, b*), chroma and hue-angle of the control oil (ie. neutralised oil B_0) and bleached oils (B_{1-5}). The treatments showed more than 75 % recovery after bleaching with respect to the neutralized oil (B_0). Although the recovery was higher when treated with kaolin, cellulose and chitin (> 94 %), the quality parameters of the oil after bleaching with the adsorbents apparently indicated that they were not effective in bleaching the crude sardine oil. AC and FE when added individually reduced the FFA content of the control oil by 13.1 and 1.56 % (% reduction), respectively. Hence, the removal of the soap stock residues in the neutralized oil was evident (Rossi *et al*., 2003) using these adsorbents. The neutralized oil presented an initial P.V. of 6.1 meqO_2/kg and a pA.V. of 10, resulting in a TOTOX value of 22.2 (Table 4.6). The reduction in initial P.V. and pA.V. values while using AC (reduction of 28.2 & 5.9 %, respectively) and FE (17.6 & 4.9 %, respectively) individually were significantly higher ($p<0.05$), which denote the capacity of these adsorbents to adsorb the primary and secondary oxidation products or similar compounds, as also supported by earlier studies (Rossi *et al*., 2003; Huang & Sathivel 2010). The hydrogen-bonding ability of the carbonyl group to the surface silanol groups (Si-OH) in the Fuller's Earth might cause the reduction in the P.V. and pA.V. to a good extent. The crude oil bleached with AC and FE apparently indicated the less formation of TBA reactive species with 8.9 & 9.5 % reduction,

respectively in that order from initial value, as compared with other adsorbents used in this study. The Fuller's Earth structure consists of silica, alumina and monovalent and bivalent cations located between the layers of silica and alumina. The molecular configuration allows the FE for use as an efficient adsorbent to absorb the impurities in the fish oil component. Primary and secondary oxidation products are likely to affect the color and turbidity of fish oil, if there is a higher content of primary and secondary oxidation products in fish oil, the appearance of the observed fish oil would be dark, and therefore, the level of clarity tends to decrease (Estiasih 2009).

The initial L*, a*, and b* values of the neutralized oil were found to be 51.34, 8.21, and 43.65, respectively (Table 4.6). These CIELAB coordinates denote a dark brown color, which was mostly due to pigments such as carotenoids (Indrasena & Barrow 2010). The bleaching with activated charcoal and Fuller's Earth increased the lightness of the oil, reaching L* values up to 75.1 for activated charcoal (5%, 10 min) and 70.7 for Fuller's Earth (5%). Besides, the adsorption process effectively reduced a* value (3.65 for activated charcoal 5%; 3.66 for Fuller's Earth 5%) indicating a decrease in red pigment. As a result, the oil samples became lighter and slightly more transparent. The b* value of the bleached oils suffered a reduction when AC and FE were used (38.85 & 39.89, respectively). The chroma values of the bleached oils, which represent the intensity of color, were reduced, whilst the Hue-angle value increased which implies the yellow-orange color for the bleached oils.

4.2.2.2A''. Bleaching with different combinations of activated charcoal (AC) and Fuller's Earth (FE)

The effect of different combinations (BC_{1-5}) of the potent adsorbents (AC & FE) on the quality parameters of the oil was recorded in Table 4.6. The acidity of the neutralized oil reduced effectively when combinations of AC:FE with 1.25:3.75 and 4.5:0.75 (% w/w) was used.

An effective reduction of color was observed with 83.6 L^*, 3.6 a^* and 39.61 b^* for the combination of 1.25:3.75 (w/w) AC:FE (BC_4). The initial L*, a*, and b*

values of the neutralized oil were, respectively, 51.34, 8.21, and 43.65 in that order (Table 4.6). These CIELAB coordinates denote a dark brown color, which was mostly due to pigments such as carotenoids (Indrasena & Barrow 2010). The bleaching with activated charcoal and Fuller's Earth increased the lightness of the oil, reaching L* values up to 75.1 for activated charcoal (5%, 10 min) and 70.7 for Fuller's Earth (5%). Besides, the adsorption process effectively reduced a* value (3.65 for activated charcoal 5%; 3.66 for Fuller's Earth 5%) indicating a decrease in red pigment. Resultantly the oil samples became lighter and slightly more transparent. The b* value of the bleached oils showed a reduction when AC and FE were used (38.85 & 39.89, respectively). The chroma values of the bleached oils, which represent the intensity of color, were reduced (from 44.42 to 39.02 for AC and 40.06 for FE), whilst the Hue-angle value increased to 84.63 (AC) and 84.76 (FE) from 79.35 (control, B_0), respectively, which imply the yellow-orange color for the bleached oils.

4.2.2.2A'''. Bleaching with activated charcoal (AC) and Fullers earth (FE) (AC:FE 1.25:3.75, %, w/w) at different concentrations and contact time

The FFA percentage suffered a marginal increase when lesser concentration (AC:FE; 1 and 3%) of AC:FE was used. By utilizing the adsorbent concentrations at the level of 1 % (w/w), the FFA percentage experienced a general increase as compared to the FFA content at 3 and 5% adsorbent concentrations. This might be due to the hydrolysis of TAGs while heating, which lead to the formation of free fatty acids. This phenomenon was not observed by the charcoal- Fuller's Earth system, whereas the clay concentration used was 1 % (w/w). Other possible explanations include the presence of other impurities such as minerals and protein in the fish oil, or the high viscosity of the oil, resulting in the difficulty in mass transfer, and thus the final removal of FFA (Huang & Sathivel 2010). An acceptable level of FFA in refined fish oil was reported between 1.8 % and 3.5 % (Sathivel *et al*., 2003). A combination treatment in oil refining can reduce the levels of free fatty acids, primary and secondary oxidation products of the oil. This implied an increase in

clarity after fish oil bleaching. For the bleached oil, which was processed at the highest adsorbent amount (3 & 5 %), the P.V. of < 4 meqO_2/kg was obtained when less contact time was used (20 & 40 min). However, the bleaching process carried out using lower concentrations of the adsorbent (1%), and at low process temperatures, did not reduce the P.V. of the oil, but increased the formation of the peroxides (BC_{4I} > 6.3 meqO_2/kg) due to the exposure of the oil to heat for more contact time (60 min). The bleached oils when processed using the highest amount of the adsorbent (5%), no significant change of P.V. was discernable as compared with that by using 3% concentration of the adsorbent. Nevertheless, the bleaching process carried out using lower concentrations of AC:FE (i.e., at 1%), and at low process temperatures, did not reduce the P.V. of the oil; on the contrary, these conditions increased the formation of peroxides due to the exposure of the oil to high temperatures. It is of note that the P.V. was recorded below the threshold level for human consumption of 8 meqO_2/kg oil (Boran *et al*., 2006), and the crude oils provided a good indices of oxidative quality. Palanisamy *et al*. (2011) showed that the absorbent bentonite was able to reduce the content of the peroxides on ostrich oil up to 93.9 with 10% bentonite concentration, while performing the adsorption process for a contact time of 70 min. Differences in the effectiveness of peroxide reduction might be caused by differences in the sample to be purified, the length of the adsorption process, adsorption temperature and concentration of adsorbent used (Patterson 2009). Considering the secondary oxidation products, the Table 4.6 shows a reduction in pA.V. in the bleached oils adsorbed with 3-5% concentration. Consequently it is evident that within the experimental conditions, the adsorption of secondary oxidation products was more effective at higher concentration of adsorbents and reached the optimum even at 3% instead of 5% level of the adsorbents used. For the adsorbent amount of 5% w/w with stirring of 40 min, the peroxide value of 3.92 meqO_2/kg was noted. An acceptable pA.V. for good-quality crude fish oils was reported to be lower than 20 (Hamilton *et al*., 1988). As represented in the Table 4.6, the lowest pA.V. values presented for bleached oils was

at 3 and 5% adsorbent concentration for 40 min contact time (9.4). However, in order to optimize the quality of the bleached oils in terms of oxidation products, a minimum TOTOX value was required, which in the present study was achieved by using the adsorbent at 3 and 5% for 40 min contact time (BC_{4B} & BC_{4E}, respectively 9.4). The total oxidation level was found to be increased with a decrease in the adsorbent concentration, from 3 to 1%, although the total oxidation increased while the adsorbent was used at 5% for 60 min contact time (18.6). It is strongly associated with an inclining trend due to peroxide oxidation, whereas the total oxidation was determined as twice the peroxide value (2 x P.V.). Reduction of the total oxidation in the adsorption process with 3% AC:FE for 40 min contact time was recorded as 24.7 %. However, higher adsorbent concentration (5%) might allow maximum adsorption of impurities, while enabling the natural antioxidants contained in the pigment to be adsorbed thereby affecting the stability of the fish oil. An earlier study demonstrated that the treatment using bleaching earth can absorb up to half of the natural antioxidants in the oil (Patterson 2009). In addition, List et al. (1972) also reported that sunflower oils treated in the laboratory with a high concentration of bleaching earth (6%) had poorer flavor and oxidative stability than the same oil bleached with a lower concentration of activated earth (2%). It corroborates well with the present study from the low TOTOX values of the treatments BC_{4D-F} when a concentration of 3 % adsorbent was used**,** as compared to 5 % concentration of the adsorbent (BC_{4A-C}) (Table 4.6). The TBA values recorded for the bleached oils (Table 4.6) is in good agreement with the optimum patterns for oil quality and acceptability for human consumption, as reported in an earlier study (7–8 mg malonaldehyde/kg oil) (Boran *et al*., 2006).

The highest L* value was achieved at both 5% and 3% concentration (both 83.74) with 40 min contact time. The increased L* value during fish oil adsorbent purification process also had been reported by Huang and Sathivel (2010), which resulted the increment of the L* value from 27 to 34.6 while using chitosan and from 27-42.1 with activated earth during the purification process of the salmon oil.

According to Gauglitz and Gruger (1965), the adsorbents remove the suspended mucilaginous and colloid-like matter so the brightness of the oil could be improved. The lower a* value of bleached oil reached ≤ 3.64 for 3 and 5% concentrations (BC_{4A-F}) indicating a decrease in the red pigmentation pattern. As a consequence, the oil samples became lighter and more transparent. However, a lower adsorbent concentration (1%) did not effectively reduce the a* value (> 5). Hence the increasing of concentration of the adsorbent and adsorption time result more greenish color to sardine oil (a* < 3.64). The greenish color in the oil was produced due to the reduction of red pigment during the process of adsorption. Earlier study in salmon oil revealed the reduction of carotenoid pigments such as astaxanthin and canthaxanthin during adsorption with a decrease in a* value (redness), and increase in –a* value (greenness) while using chitosan and activated earth as adsorbents (Huang & Sathivel 2010). The initial value of b* was found to be as 43.65, and after the adsorption process the b* value reached ≤ 38 (13 % improvement) at 3 and 5% level of concentrations, respectively, which denote the yellowish color. The chroma values of the bleached oils, which represent the intensity of color, were found to be reduced (≤ 38), whilst, the hue-angle value increased with the concentration of the adsorbent. The optimum value of hue angle was found at the high adsorbent concentrations (3 & 5 %).

A 3% concentration of the combination (activated charcoal: Fuller's Earth, 1.25:3.75) with 40 min of stirring (BC_{4E}) was observed to be optimum for decolorization process. The reductions in oxidation parameters of the bleached oil implied that there is an improvement in the quality of the oil, as it reduced the vulnerability of the oil towards rancidity and thereby improve stability. In addition, the color of the oil was observed to be golden brown, which indicate the significance of the percentage pigments removed.

Table 4.6 Characteristics of sardine oil after different modes of bleaching

		Yield (%)	FFA	P.V	pA.V	TOTOX	TBARS	L*	a*	b*	Chroma	Hue angle	ΔE
B_0	Control	80.36(93.44)	3.21±0.32^a	6.09±0.61^a	10.01±1^a	22.19±2.22^a	4.02±0.4^a	51.34±5.13^a	8.21±0.82^a	43.65±4.37^a	44.42±4.44^a	79.35±7.94^a	
Different adsorbents added to B_0													
	Adsorbent concentration												
B_1	AC(5)	75.41(93.84)	2.79±0.29^a	4.37±0.44^{aa}	9.42±0.94^a	18.16±1.82^{aa}	3.66±0.37^a	75.06±1.51^a	3.65±0.37^a	38.85±3.89^{aa}	39.02±3.9^{aa}	84.63±1.46^a	24.63±2.45^a
B_2	KA(5)	78.54(97.74)	3.37±0.34^a	6.45±0.65^a	10.45±1.05^a	23.35±2.35^a	4.12±0.41^a	51.06±0.11^a	6.52±0.65^a	41.69±4.17^a	42.2±4.22^a	81.1±0.11^a	2.6±0.26^a
B_3	FE(5)	77.65(96.63)	3.16±0.32^a	5.02±0.5^a	9.52±0.95^a	19.56±1.95^a	3.64±0.36^a	70.74±1.08^a	3.66±0.37^a	39.89±1.99^{aa}	40.06±4.01^{aa}	84.76±2.48^a	20.28±2.03^a
B_4	CE(5)	78.01(97.08)	3.41±0.34^a	6.03±0.6^a	9.91±0.99^a	21.97±1.19^a	4.01±0.4^a	51.42±1.14^a	7.52±0.75^{aa}	40.64±0.06^{aa}	41.33±4.13^a	79.51±1.95^a	3.09±0.31^a
B_5	CH(5)	79.01(98.32)	3.39±0.34^a	5.73±0.58^a	9.94±0.99^a	21.4±2.15^a	4.01±0.4	51.45±2.15	4.99±0.5	40.61±0.06	40.92±4.09	83±8.3	4.43±0.45
Combinations of AC & FE (total 5%)													
BC_1	AC:FE(2.5:2.5)	75.69(94.19)	3.18±0.31^a	4.67±0.47^a	9.47±0.95^a	18.81±1.89^{aa}	3.67±0.37^a	75.28±1.53^a	3.67±0.37^a	39.96±4^{aa}	40.13±4.01^{aa}	84.75±1.48^a	24.64±0.46^a
BC_2	AC:FE(4.5:0.5)	75.88(94.43)	2.74±0.27^a	4.56±0.47^{ac}	9.47±0.95^a	18.59±1.89^{aa}	3.68±0.37^a	75.23±3.52^a	3.65±0.37^a	39.78±2.98^{aa}	39.95±0.04^{aa}	84.75±0.48^a	24.63±1.46^a
BC_3	AC:FE(3.75:1.25)	75.54(94)	2.94±0.29^a	4.95±0.5^a	9.3±0.93^a	19.2±1.93^a	3.5±0.35^a	74.16±0.42^a	3.61±0.36^a	39.86±3.99^{aa}	40.02±.4^{aa}	84.82±3.48^a	23.59±1.36^a
BC4	AC:FE(1.25:3.75)	74.12(92.23)	2.79±0.27^a	4.3±0.43^{aa}	9.42±0.94^a	18.02±1.8^{aa}	3.64±0.36^a	83.57±2.35^a	3.6±0.36^a	39.61±1.96^{aa}	39.77±3.98^{aa}	84.8±0.48^a	32.81±2.29^c
BC_5	AC:FE(0.5:4.5)	75.14(93.5)	3.01±0.3^a	5.08±0.5^a	9.3±0.93^a	19.46±1.93^a	3.68±0.37^a	81.51±0.15^a	3.61±0.36^a	39.24±0.93^{aa}	39.41±1.94^{aa}	84.74±1.47^a	30.84±1.08^c
Combination BC4 at different concentrations (%) and contact time (min)													
	Contact time (min)												
BC_{4A}(5%)	20	75.14(93.5)	2.79±0.29^a	3.62±0.36^a	9.49±0.95^a	16.73±1.67^a	3.67±0.37^a	83.54±1.35^a	3.6±0.36^a	37±0.7^a	37.17±3.72^a	84.44±2.44^a	91.44±1.14^d
BC_{4B}(5%)	40	74.12(92.23)	2.78±0.28^a	3.92±0.39^{ac}	9.4±0.94^a	17.24±1.72^a	3.75±0.38^a	83.74±8.37^a	3.64±0.36^a	37.02±3.7^a	37.2±3.72^a	84.38±0.45^a	91.63±0.16^d
BC_{4C}(5%)	60	72.15(89.78)	2.78±0.28^a	4.15±0.42^{ac}	10.34±1.03^a	18.64±1.87^{aa}	4.08±0.41^a	81.89±2.19^a	3.62±0.36^a	37.16±2.71^a	37.34±3.73^a	84.44±1.44^a	90±0.09^d
BC_{4D}(3%)	20	74.98(93.31)	2.74±0.27^a	3.92±0.39^{ac}	9.22±0.92^a	17.06±1.7^a	3.67±0.37^a	81.9±0.19^a	3.56±0.36^a	38.01±3.8^a	38.18±3.82^a	84.65±0.47^a	90.36±1.04^d
BC_{4E}(3%)	40	74.78(93.06)	2.94±0.3^a	3.65±0.38^a	9.4±0.94^a	16.7±1.7^a	3.62±0.36^a	83.74±0.37^a	3.61±0.36^a	37.99±3.8^a	38.16±1.82^a	84.57±3.47^a	92.03±0.2^d
BC_{4F}(3%)	60	73.65(91.65)	2.76±0.28^a	4.13±0.41^{ac}	9.87±0.99^a	18.13±1.81^{aa}	4.56±0.46^a	83.73±8.37^a	3.54±0.35^a	37.56±3.77^a	37.73±3.77^a	84.61±1.46^a	91.84±2.18^d
BC_{4G}(1%)	20	72.51(90.23)	2.96±0.3^a	5.09±0.51^a	10.45±1.05^a	20.63±2.07^a	3.89±0.39^a	81.63±8.16^a	5.22±0.52^{aa}	39.94±0.98^{aa}	40.28±0.03^{aa}	82.55±3.26^a	91.03±4.1^d
BC_{4H}(1%)	40	71.25(88.66)	2.97±0.3^a	5.15±0.52^a	10.89±1.09^a	21.19±2.13^a	4.08±0.41^a	81.36±8.14^a	5.21±0.52^{aa}	39.28±3.93^{aa}	39.62±3.97^{aa}	82.44±2.25^a	90.5±2.05^d
BC_{4I}(1%)	60	70.31(87.49)	3.14±0.3^a	6.29±0.64^a	11.25±1.13^a	23.83±2.41^a	5.08±0.51^a	81.34±8.13^a	5.4±0.54^{aa}	39.91±1.99^{aa}	40.27±1.03^{aa}	82.29±1.23^a	90.76±3.08^d

B_0 - Neutralized oil; AC – activated charcoal; KA – kaolin; FE – fullers earth; CE – cellulose powder; CH –chitin; Data are expressed as mean ± standard deviation of three replicates. Means with different superscripts (a, b, c) in the row indicates a statistical difference ($p<0.05$). Other notations are as represented in Materials and Methods section.

4.2.2.2B. Fatty Acid Composition of the Bleached Sardine Oil

The fatty acid profiles of the sardine oil after bleaching experiments have been recorded in Table 4.7.1 (B_0-B_5), 4.7.2 (BC_1-BC_5) and 4.7.3 (BC_{4A-I}). A significant reduction in ∑PUFA and *n*-3 PUFA were observed for B_4 and B_5 (≤ 20 & < 17 %, respectively, $p<0.05$). For B_2 – B_5, the SFAs were found to be significantly higher than the untreated neutralized oil (B_0) ($p<0.05$). The reduction in EPA and DHA were recorded to be at their minimum for the treated oils. The *trans* fatty acid content was also considerably reduced in B_3 (to 0.04 % from 0.09 %).

The combinations of AC and FE showed a total of 38.9 – 44.8 % ∑SFA (Table 4.7.2). The ∑PUFA content increased for BC_3 and BC_4 (more than 27 %). A reduction in ∑PUFA content was observed for BC_1 and BC_3 along with a significant reduction in *trans* fatty acids for BC_3 and BC_4.

When contact time was increased from more than 10 min, the contents of ∑SFA were found to be significantly reduced (Table 4.7.3). A marginal reduction in the contents of *n*-3 PUFAs and ∑PUFA were evident for BC_{4A-C} and BC_{4G-I}. The chromatograms were shown in Fig. 4.11A-D.

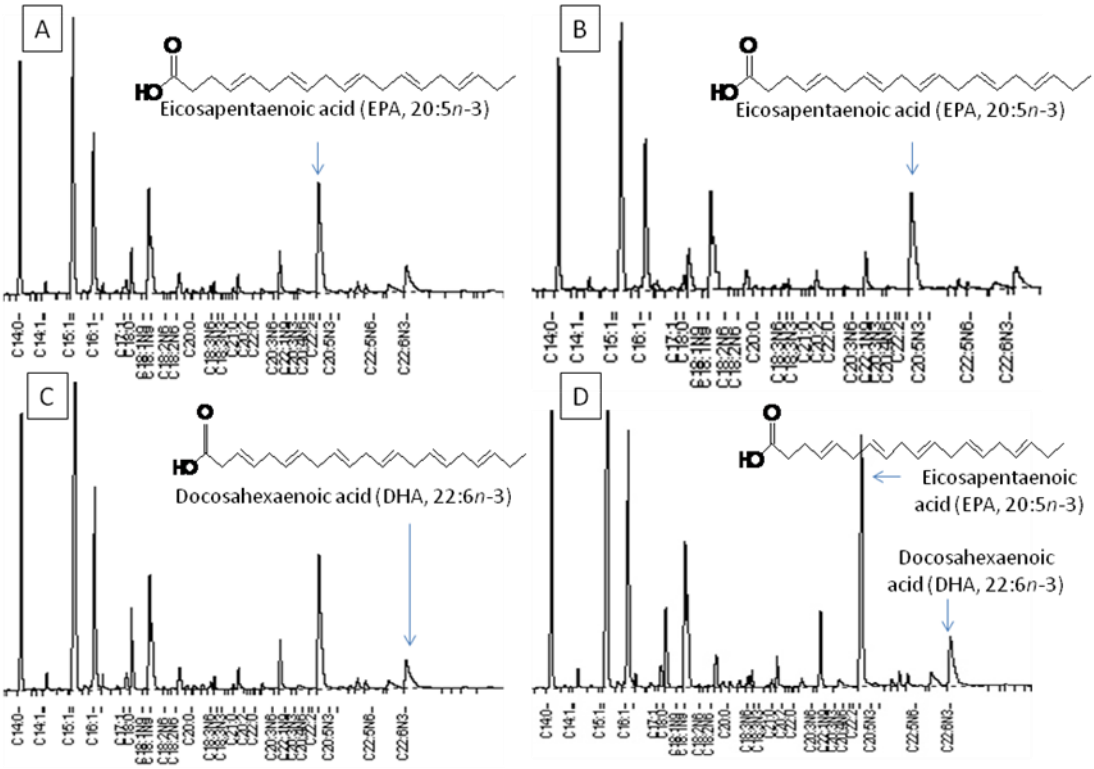


Fig. 4.9. Fatty acid chromatogram of bleached oil (**A**) B_1; (**B**) B_3; (**C**) BC_4 and (**D**)BC_{4E}

Table 4.7.1 Fatty acid composition of sardine oil after bleaching (B_0-B_5)

	B_0	B_1	B_2	B_3	B_4	B_5
Saturated						
14:0	12.28±1.23^a	12.5±1.25^a	13.16±1.32^a	11.63±1.15^a	12.39±1.24^a	11.19±1.11^a
15:0	0.76±0.08^a	0.7±0.07^a	0.77±0.08^a	0.71±0.07^a	0.9±0.09^a	0.74±0.07^a
16:0	20.94±1.08^a	21.15±2.12^a	26.21±2.62^{ab}	27.92±2.79^b	34.83±0.48^c	32.64±3.26^c
17:0	0.69±0.07^a	0.68±0.07^a	0.78±0.08^a	0.77±0.08^a	0.49±0.05^a	0.79±0.08^a
18:0	4.81±0.48^a	4.2±0.42^a	5.58±0.56^a	5.71±0.55^a	7.85±0.79^a	6.76±0.67^a
20:0	0.39±0.04^a	0.27±0.03^a	0.37±0.04^a	0.3±0.03^a	0.5±0.05^a	0.27±0.03^a
22:0	0.29±0.03^a	0.44±0.04^a	0.41±0.04^a	0.39±0.04^a	0.63±0.06^a	0.35±0.04^a
24:0	0.03±0^a	0.06±0.01^a	0.01±0^a	0.12±0.01^a	0.14±0.01^a	0.1±0.01^a
∑SFA	40.18±1.01^a	40±0.01^a	47.29±2.74^b	47.55±1.72^b	57.73±2.77^c	52.84±0.27^{bc}
Monounsaturated						
14:1*n*-7	0.07±0.01^a	0.09±0.01^a	0.08±0.01^a	0.07±0.01^a	0.05±0.01^a	0.07±0.01^a
15:1*n*-7	0.18±0.02^a	0.03±0^a	0.01±0^a	0.01±0^a	0.01±0^a	0.11±0.01^a
16:1*n*-7 *trans*	0.03±0^a	0.03±0^a	0.02±0^a	0.02±0^a	0.04±0^a	0.03±0^a
16:1*n*-7	13.41±1.65^a	12.83±1.28^a	12.29±1.23^a	11.22±1.12^a	9.4±0.94^a	10.3±1.02^a
18:1*n*-9 *trans*	0.03±0^a	0.02±0^a	0.02±0^b	0.01±0^a	0.03±0^b	0.02±0^{ab}
18:1*n*-9	10.49±1.05^a	11.96±1.2^a	6.78±0.68^a	10.12±1.01^a	5.4±0.54^a	9.19±0.92^a
20:1*n*-9	0.61±0.06^a	0.67±0.07^a	0.52±0.05^a	0.57±0.06^a	0.52±0.05^a	0.38±0.04^a
22:1*n*-9	3.1±0.3^a	3.18±0.32^a	3.01±0.3^a	2.72±0.27^a	2.42±0.24^a	2.47±0.25^a
24:1*n*-9	1.05±0.11^a	0.36±0.04^a	0.27±0.03^a	0.4±0.04^a	0.48±0.05^a	0.3±0.03^a
∑MUFA	28.98±0.2^a	29.17±2.92^a	23±1.03^{bc}	25.14±2.51^{ab}	18.35±0.83^c	22.87±2.28^{bc}
Polyunsaturated						
18:2*n*-6 *trans*	0.03±0^a	0.03±0^a	0.03±0^a	0.01±0^a	0.02±0^a	0.02±0^a
18:2*n*-6	1.89±0.19^a	1.87±0.19^a	1.89±0.19^a	1.63±0.16^a	1.4±0.14^a	1.47±0.15^a
18:3*n*-6	0.44±0.04^a	0.52±0.05^a	0.41±0.04^a	0.43±0.04^a	0.41±0.04^a	0.34±0.03^a
18:3*n*-3	0.71±0.07^a	0.3±0.03^a	0.35±0.04^a	0.23±0.02^a	0.3±0.03^a	0.24±0.02^a
20:2*n*-6	1.26±0.13^a	1.24±0.12^a	1.25±0.12^a	0.92±0.09^a	0.94±0.09^a	0.84±0.08^a
20:3*n*-6	0.13±0.01^a	0.08±0.01^a	0.07±0.01^a	0.11±0.01^a	0.04±0^a	0.07±0.01^a
20:4*n*-6	0.09±0.01^a	0.61±0.06^a	0.53±0.05^a	0.54±0.05^a	0.45±0.05^a	0.47±0.05^a
20:5*n*-3 EPA	15.02±1.5^a	15.04±1.5^a	15.01±1.4^a	14.05±1.41^a	11.31±1.13^a	11.89±1.19^a
22:5*n*-3	0.7±0.07^a	0.77±0.08^a	0.49±0.05^a	0.73±0.07^a	0.49±0.05^a	0.57±0.06^a
22:6*n*-3 DHA	5.28±0.53^a	5.34±0.53^a	5.09±0.5^a	4.95±0.5^a	3.92±0.39^a	4.09±0.4^a
∑PUFA	25.55±2.55^a	25.8±2.57^a	25.12±2.4^a	23.6±2.35^a	19.28±1.92^b	20±1.99^{ab}
EPA+DHA	20.3±2.03^a	20.38±2.03^a	20.1±1.9^a	19±1.91^a	15.23±1.52^b	15.98±1.59^b
∑*n*-3PUFA	21.71±0.17^a	21.45±0.14^a	20.94±1.99^a	19.96±0.02^a	16.02±1.6^a	16.79±1.67^a
∑n-6PUFA	3.81±0.38^a	4.32±0.43^a	4.15±0.41^a	3.63±0.35^a	3.24±0.32^a	3.19±0.32^a
∑*n*-3/∑*n*-6	5.7±1.71^a	4.97±1.98^a	5.05±0.85^a	5.5±1.71^a	4.94±5^a	5.26±5.22^a
∑*n*-6/∑*n*-3	0.18±0.18^a	0.2±0.2^a	0.2±0.21^a	0.18±0.18^a	0.2±0.2^a	0.19±0.19^a
∑PUFA/∑SFA	0.64±0.64^a	0.65±0.64^a	0.53±0.51^a	0.5±0.5^a	0.33±0.33^a	0.38±0.38^a
∑TRANS	0.09±0^a	0.08±0^a	0.07±0^a	0.04±0^a	0.09±0^a	0.07±0^a

B_0 - Neutralized oil; AC – activated charcoal; KA – kaolin; FE – fullers earth; CE – cellulose powder; CH –chitin; Data are expressed as mean ± standard deviation of three replicates. Means with different superscripts (a, b etc) in the row indicates a statistical difference ($p<0.05$). Other notations are as represented in Table 4.3.

Table 4.7.2. Fatty acid composition of sardine oil after bleaching with different concentrations of activated charcoal and fullers earth (BC_1-BC_5)

	BC_1	BC_2	BC_3	BC_4	BC_5
Saturated					
14:0	12.58±1.26	14.14±1.41	12.85±1.29	13.88±1.39	12.23±1.22
15:0	0.68±0.07	0.72±0.07	0.74±0.07	0.75±0.08	0.68±0.07
16:0	21.58±2.16	18.81±1.88	24.87±2.49	23.46±2.35	20.88±1.09
17:0	0.68±0.07	0.83±0.08	0.76±0.08	0.73±0.07	0.65±0.07
18:0	4.17±0.42	3.82±0.38	4.81±0.48	4.48±0.45	4.08±0.41
20:0	0.26±0.03	0.33±0.03	0.34±0.03	0.37±0.04	0.27±0.03
22:0	0.42±0.04	0.19±0.02	0.45±0.05	0.26±0.03	0.44±0.04
24:0	0.09±0.01	0.08±0.01	0.04±0	0.05±0.01	0.05±0.01
∑SFA	40.46±1.05	38.92±0.89	44.86±2.49	43.98±0.04	39.28±0.93
Monounsaturated					
14:1*n*-7	0.14±0.01	0.08±0.01	0.07±0.01	0.07±0.01	0.08±0.01
15:1*n*-7	0.02±0	0.13±0.01	0.01±0	0.12±0.01	0.11±0.01
16:1*n*-7 *trans*	0.02±0	0.03±0	0.01±0	0.01±0	0.01±0
16:1*n*-7	12.98±1.3	15.31±1.53	12.82±1.28	14.14±1.41	12.51±1.25
18:1*n*-9 *trans*	0.03±0	0.04±0	0.01±0	0.01±0	0.02±0
18:1*n*-9	11.66±1.17	8.99±0.9	6.7±0.67	5.59±0.56	11.55±1.16
20:1*n*-9	0.5±0.05	0.67±0.07	0.68±0.07	0.52±0.05	0.65±0.07
22:1*n*-9	3.21±0.32	2.76±0.28	3.28±0.33	3.34±0.33	3.09±0.31
24:1*n*-9	0.28±0.03	0.16±0.02	0.35±0.04	0.17±0.02	0.5±0.05
∑MUFA	28.84±1.88	28.17±2.82	23.93±0.39	23.97±2.4	28.52±2.85
Polyunsaturated					
18:2*n*-6 *trans*	0.03±0	0.04±0	0.02±0	0.02±0	0.02±0
18:2*n*-6	1.88±0.19	1.98±0.2	1.88±0.19	2.03±0.2	1.81±0.18
18:3*n*-6	0.45±0.05	0.65±0.07	0.54±0.05	0.47±0.05	0.52±0.05
18:3*n*-3	0.29±0.03	0.29±0.03	0.29±0.03	0.24±0.02	0.32±0.03
20:2*n*-6	1.27±0.13	1.26±0.13	1.08±0.11	1.38±0.14	1.2±0.12
20:3*n*-6	0.08±0.01	0.31±0.03	0.12±0.01	0.02±0	0.11±0.01
20:4*n*-6	0.65±0.07	0.49±0.05	0.61±0.06	0.28±0.03	0.61±0.06
20:5*n*-3 EPA	15.05±1.51	13.55±1.36	15.52±1.55	16.31±1.63	14.58±1.46
22:5*n*-3	0.71±0.07	0.2±0.02	0.7±0.07	0.77±0.08	0.76±0.08
22:6*n*-3 DHA	5.09±0.51	4.23±0.42	6.25±0.63	5.91±0.59	5.2±0.52
∑PUFA	25.5±2.55	23±2.3	27.01±2.7	27.43±2.74	25.13±1.51
EPA+DHA	20.14±2.01	17.78±1.78	21.77±2.18	22.22±2.22	19.78±1.98
∑*n*-3PUFA	21.14±2.11	18.27±1.83	22.76±2.28	23.23±2.32	20.86±0.09
∑n-6PUFA	4.33±0.43	4.69±0.47	4.23±0.42	4.18±0.42	4.25±0.43
∑*n*-3/∑*n*-6	4.88±0.49	3.9±0.39	5.38±0.54	5.56±0.56	4.91±0.49
∑*n*-6/∑*n*-3	0.2±0.02	0.26±0.03	0.19±0.02	0.18±0.02	0.2±0.02
∑PUFA/∑SFA	0.63±0.06	0.59±0.06	0.6±0.06	0.62±0.06	0.64±0.06
∑TRANS	0.08±0.01	0.11±0.01	0.04±0	0.04±0	0.05±0.01

Data are expressed as mean ± standard deviation of three replicates. No significant differences were observed between the five variants ($p>0.05$). Other notations are as represented in Table 4.3.

Table 4.7.3. Fatty acid composition after bleaching with 1.25:3.75 (% w/w) AC:FE at different concentrations and contact time

	BC_{4A}	BC_{4B}	BC_{4C}	BC_{4D}	BC_{4E}	BC_{4F}	BC_{4G}	BC_{4H}	BC_{4I}
Saturated									
14:0	10±1	9.96±1	10.64±1.06	10.84±1.08	11.62±1.16	11.04±1.1	12.01±1.2	12.3±1.23	11.56±1.16
15:0	0.58±0.06	0.57±0.06	0.59±0.06	0.68±0.07	0.66±0.07	0.68±0.07	0.69±0.07	0.7±0.07	0.7±0.07
16:0	20.01±0.12	19.63±1.96	19.86±1.99	19.76±1.98	20.11±2.01	20.11±0.01	20.26±2.03	20.92±2.09	21.01±2.1
17:0	0.56±0.06	0.57±0.06	0.57±0.06	0.66±0.07	0.64±0.06	0.65±0.07	0.67±0.07	0.64±0.06	0.64±0.06
18:0	3.26±0.33	3.52±0.35	3.67±0.37	3.79±0.38	4.29±0.43	4.08±0.41	4.11±0.41	4.11±0.41	5.12±0.51
20:0	0.26±0.03	0.24±0.02	0.25±0.03	0.31±0.03	0.32±0.03	0.33±0.03	0.31±0.03	0.29±0.03	0.29±0.03
22:0	0.38±0.04	0.37±0.04	0.46±0.05	0.47±0.05	0.5±0.05	0.46±0.05	0.44±0.04	0.41±0.04	0.46±0.05
24:0	0.1±0.01	0.09±0.01	0.08±0.01	0.08±0.01	0.07±0.01	0.08±0.01	0.11±0.01	0.13±0.01	0.13±0.01
∑SFA	35.15±0.52	34.95±3.5	36.12±2.61	36.59±3.66	38.21±3.82	37.43±1.74	38.6±3.86	39.5±3.95	39.91±0.99
Monounsaturated	0.01±0	0.06±0.01	0.05±0.01	0.05±0.01	0.06±0.01	0.07±0.01	0.11±0.01	0.15±0.02	0.15±0.02
14:1*n*-7									
15:1*n*-7	0.01±0	0.01±0	0.02±0	0.01±0	0.01±0	0.07±0.01	0.04±0	0.04±0	0.04±0
16:1*n*-7 *trans*	0.01±0	0.01±0	0.01±0	0.01±0	0.01±0	0.02±0	0.03±0	0.02±0	0.02±0
16:1*n*-7	11.56±1.16	11.47±1.15	11.86±1.19	12.11±1.21	12.44±1.24	12.01±1.2	12.16±1.22	12.66±1.27	12.55±1.26
18:1*n*-9 *trans*	0.01±0	0.01±0	0.03±0	0.01±0	0.02±0	0.03±0	0.02±0	0.02±0	0.02±0
18:1*n*-9	10.81±1.08	10.72±1.07	10.84±1.08	11.64±1.16	11.54±1.15	11.66±1.17	11.24±1.12	11.67±1.17	11.68±1.17
20:1*n*-9	0.56±0.06	0.57±0.06	0.65±0.07	0.66±0.07	0.64±0.06	0.68±0.07	0.66±0.07	0.66±0.07	0.66±0.07
22:1*n*-9	2.58±0.26	2.59±0.26	3.11±0.31	3.03±0.3	3.23±0.32	3.12±0.31	3.13±0.31	3.1±0.31	3.01±0.3
24:1*n*-9	0.37±0.04	0.36±0.04	0.56±0.06	0.55±0.06	0.54±0.05	0.34±0.03	0.54±0.05	0.32±0.03	0.33±0.03
∑MUFA	25.92±2.59	25.8±2.58	27.13±2.71	28.07±2.81	28.49±2.85	28±2.8	27.93±2.79	28.64±2.86	28.46±1.85
Polyunsaturated									
18:2*n*-6 *trans*	0.01±0	0.01±0	0.01±0	0.01±0	0.02±0	0.01±0	0.02±0	0.02±0	0.02±0
18:2*n*-6	1.54±0.15	1.5±0.15	1.56±0.16	1.67±0.17	1.87±0.19	1.86±0.19	1.87±0.19	1.84±0.18	1.97±0.2
18:3*n*-6	0.42±0.04	0.41±0.04	0.42±0.04	0.52±0.05	0.56±0.06	0.55±0.06	0.54±0.05	0.51±0.05	0.51±0.05
18:3*n*-3	0.26±0.03	0.25±0.03	0.23±0.02	0.31±0.03	0.29±0.03	0.32±0.03	0.34±0.03	0.31±0.03	0.31±0.03
20:2*n*-6	1.01±0.1	1±0.1	1.01±0.1	1.01±0.1	1.26±0.13	1.02±0.1	1.21±0.12	1.21±0.12	1.22±0.12
20:3*n*-6	0.06±0.01	0.07±0.01	0.08±0.01	0.07±0.01	0.06±0.01	0.07±0.01	0.05±0.01	0.16±0.02	0.16±0.02
20:4*n*-6	0.52±0.05	0.51±0.05	0.56±0.06	0.78±0.08	0.76±0.08	0.54±0.05	0.54±0.05	0.58±0.06	0.58±0.06
20:5*n*-3 EPA	14.26±1.43	14.18±1.42	14.08±1.41	15.26±1.53	15.25±1.53	15.01±1.5	15.02±1.5	14.49±1.45	15±1.5
22:5*n*-3	0.72±0.07	0.71±0.07	0.76±0.08	0.77±0.08	0.75±0.08	0.76±0.08	0.71±0.07	0.72±0.07	0.72±0.07
22:6*n*-3 DHA	5.86±0.59	5.85±0.59	5.75±0.58	5.82±0.58	5.81±0.58	5.06±0.51	5.11±0.51	5.18±0.52	5.21±0.52
∑PUFA	24.66±2.47	24.49±2.45	24.46±2.45	26.22±2.62	26.63±2.66	25.2±2.52	25.41±2.54	25.02±2.5	25.7±2.57
EPA+DHA	20.12±2.01	20.03±2	19.83±1.98	21.08±2.11	21.06±2.11	20.07±2.01	20.13±2.01	19.67±1.97	20.21±2.02
∑*n*-3PUFA	21.1±2.11	20.99±2.1	20.82±2.08	22.16±2.22	22.1±2.21	21.15±2.12	21.18±2.12	20.7±2.07	21.24±2.12
∑n-6PUFA	3.55±0.36	3.49±0.35	3.63±0.36	4.05±0.41	4.51±0.45	4.04±0.4	4.21±0.42	4.3±0.43	4.44±0.44
∑*n*-3/∑*n*-6	5.94±0.59	6.01±0.6	5.74±0.57	5.47±0.55	4.9±0.49	5.24±0.52	5.03±0.5	4.81±0.48	4.78±0.48
∑*n*-6/∑*n*-3	0.17±0.02	0.17±0.02	0.17±0.02	0.18±0.02	0.2±0.02	0.19±0.02	0.2±0.02	0.21±0.02	0.21±0.02
∑PUFA/∑SFA	0.7±0.07	0.7±0.07	0.68±0.07	0.72±0.07	0.7±0.07	0.67±0.07	0.66±0.07	0.63±0.06	0.64±0.06
∑TRANS	0.03±0	0.03±0	0.05±0.01	0.03±0	0.05±0.01	0.06±0.01	0.07±0.01	0.06±0.01	0.06±0.01

Data are expressed as mean ± standard deviation of three replicates. Means with different superscripts (a, b, c) in the row indicates a statistical difference ($p<0.05$). Other notations are as represented in Table 4.3.

4.2.2.2C. Spectral Analyses of the Oil after Degumming(DG_1) and Bleaching (BC_{4E})

^{1}H-NMR spectra of the degummed sardine oil DG_1 was compared with E_6 (control) for qualitative representation of the various functionalities present in them. The integrated areas of the characteristic regions of the ^{1}H-NMR spectra of the oils studied were found to be linearly correlated with the fatty acid data, and ^{1}H-NMR was proposed as a new method for determining the degree of degumming. The characteristic fatty acid signals of E_6 as identified by ^{1}H-NMR was confirmed using literature data (Siddiqui *et al*., 2003; Vidal *et al*., 2012) and by recording spectra of standard compounds. This revealed the presence of *n*-3 fatty acids as confirmed by a sharp triplet at δ 0.974 ppm, a structural feature for methyl proton (-C**H**$_3$) in *n*-3 PUFAs (Fig. 4.10A). The signals at δ 1.18 – 1.43 could be explained due to the methylene envelope (–(C**H**$_2$)$_n$) in the long alkyl chain. The sandwiched CH_2 *bis*-allylic protons (-CH=CH-C**H**$_2$-CH=CH-) were overlapped and displayed at δ 2.74 – 2.92 ppm (multiplets). These *bis*-allylic peaks indicate the predominance of PUFA the sardine oil. The proton integral as observed by the ^{1}H-NMR analysis of DG_1 revealed significant reduction of the hydrocarbon residues compared with DG_3 and E_6 (Fig. 4.10B). The effect of degumming using the phosphoric acid was determined by the ratio of the proton integrals of the degummed oil (DG_1) to the control oil (E_6) at the region δ 0.2 – 1.8 ppm (signals exhibited by –CH_3 and –CH_2- protons), δ 1.8 – 3.0 ppm (–CH< and -C(=O)CH_3), δ 3.0 – 4.5 ppm (free –NH_2 and -OH), and δ 4.5 – 6.5 ppm (olefinic protons). A greater reduction in the proton integral was observed for DG_1 at these regions with 13.0 (δ 0.2 – 1.8 ppm), 10.3 (δ 1.8 – 3.0 ppm), 12.9 (δ 3.0 – 4.5 ppm), and 10.6 (δ 4.5 – 6.5 ppm) percent in relation to E_6. Overall, lesser intensity of the peak at δ 6 - 7 ppm for DG_1 as compared to E_6 was observed.

The extent of the bleaching process of the oil was further studied by ^{1}H-NMR spectroscopy to understand the signals and proton integrals of the conjugated olefinic double bond protons as impurities, which resonated at δ 4 - 4.5 ppm (Fig. 4.10C). The region at δ 4 – 4.5 ppm showing the peaks of conjugated double bonds were reduced for the bleached oil (BC_{4E}). A greater reduction in the proton integral in the final bleached oil (BC_{4E}) at the ^{1}H-NMR regions with 37.5 (δ 0.2 – 1.8 ppm), 25 (δ 1.8 –

3.0 ppm), 53.9 (δ 3.0 – 4.5 ppm) and 19.2 (δ 4.5 – 6.5 ppm) percent in relation to E_6 was recorded. The reduction in proton integral with respect to the degummed oil was 28.1 (δ 0.2 – 1.8 ppm), 16.3 (δ 1.8 – 3.0 ppm), 47.1 (δ 3.0 – 4.5 ppm) and 9.6 (δ 4.5 – 6.5 ppm) percent. These greater reductions in the proton integrals supported the bleaching ability of AC:FE (1.25:3.75) at 3% concentration with a stirring time of 40 min was further confirmed by their L^*, a^*, b^* values as well as the spectral scanning data (Fig. 4.11.1 & 4.11.2).

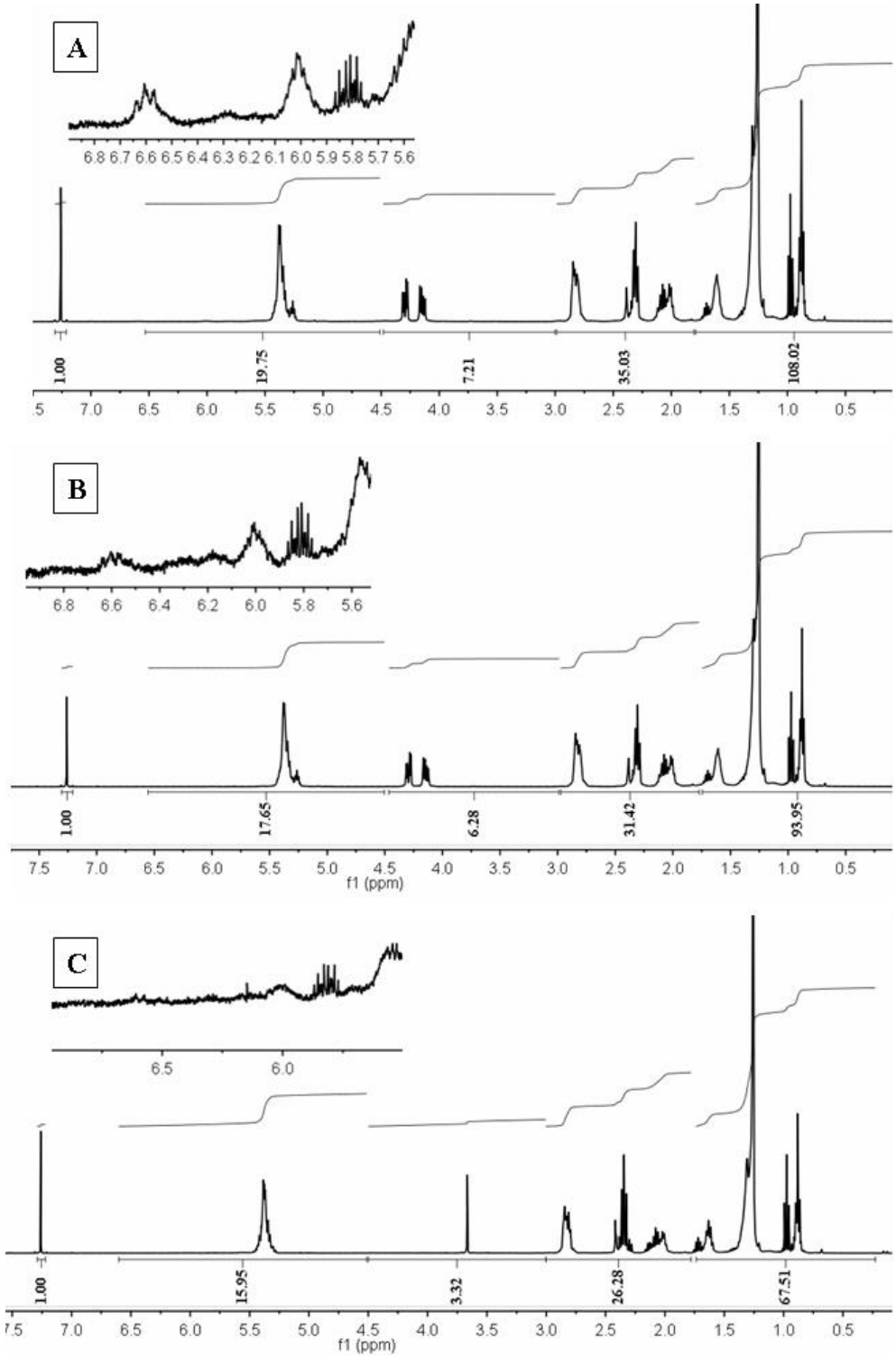


Fig. 4.10. ^{1}H-NMR spectrum of (**A**) the crude (E_6), (**B**) degummed (DG_1), and **(C)** bleached (BC_{4E}) sardine oil. The insets represent the ^{1}H NMR proton signals at δ 5.2 - 7.0 ppm.

The effective bleaching by using the optimum ratio of AC and FE (1.25:3.75) was further validated by UV-VIS scanning. The figures 4.11.1 and 4.11.2 shows indicative UV spectras after bleaching. A characteristic absorption of control oil (B_0) in the visible range was most likely caused by the chromophores with conjugated π-electron system resulting in red shifts thereby giving an undesirable color to the fish oil. The different treatments have been indicated by various colors that allow in assessing the effect of the treatment combinations to decolorize the oil. The UV-VIS spectra of the bleached oil showed a reduction in their peak intensities in the region between 340 – 370 nm (Fig. 4.11.1 & 4.11.2), which apparently signifies the reduction in the unsaturation and auxochromes bearing the electronegative groups. The bleaching process led to the removal of the color causing auxochrome and chromophoric systems in the treatment combinations. The activated charcoal adsorbs a wide range of colored impurities and contaminants by binding the impurities to the carbon. Fuller's Earth is highly adsorptive and consists of hydrated aluminium silicates. It is characterized by the property of absorbing the colors and removing them from the oils.The absorption of the fish oils in the visible regions was apparently due to the lipid-soluble pigments or pigment-like substances bearing multiple unsaturations in their structures. This indicated that fatty acid glycerides with non-conjugated double bonds did not absorb significantly in the visible region (340 -800 nm). Hence the bleaching process led to the removal of the color causing auxochrome and chromophoric systems in the AC and FE combinations.

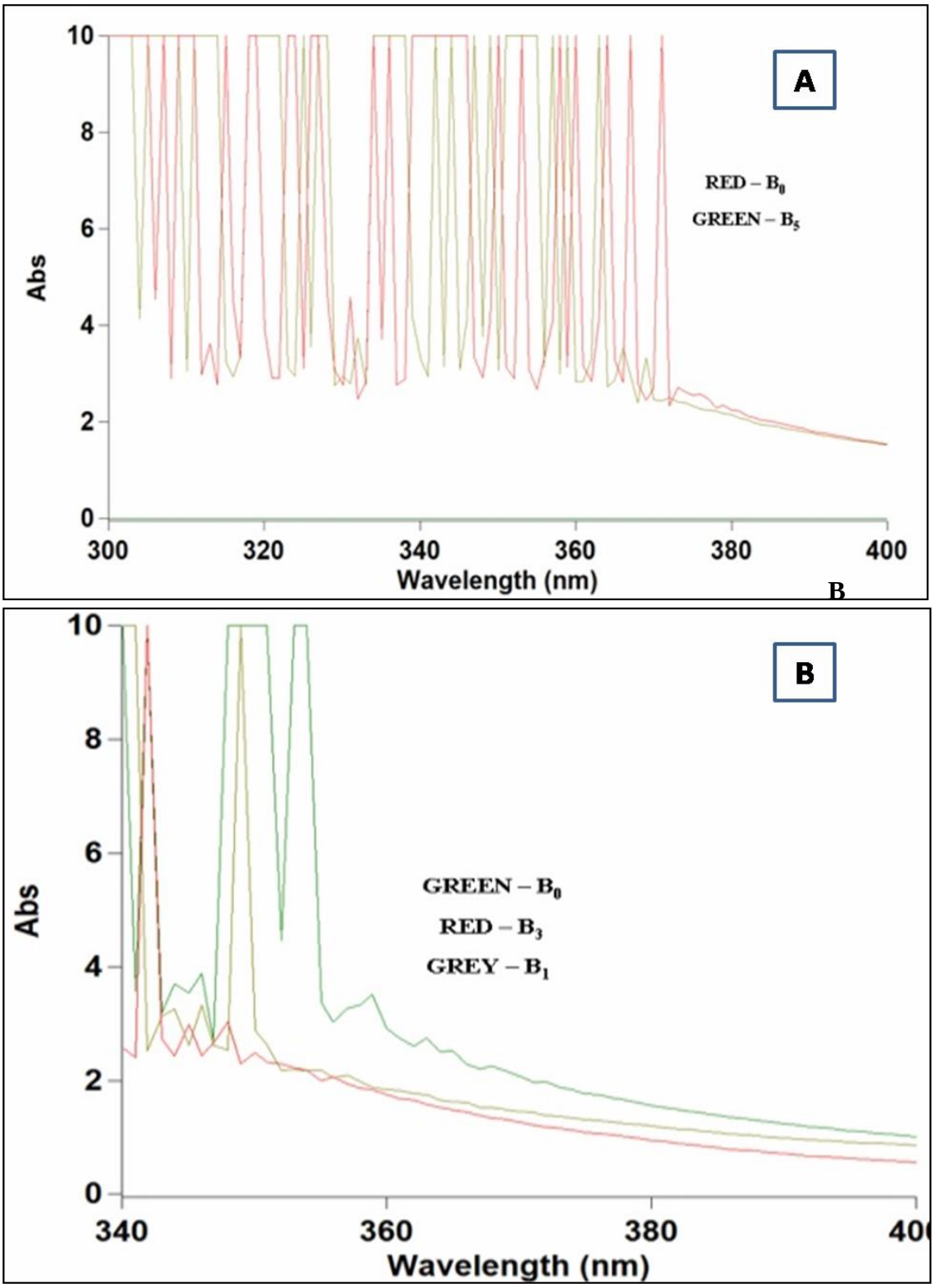


Fig.4.11.1. Absorption spectra of sardine oil before and after bleaching (**A**) B_0 & B_5; (**B**) B_0, B_1 & B_3

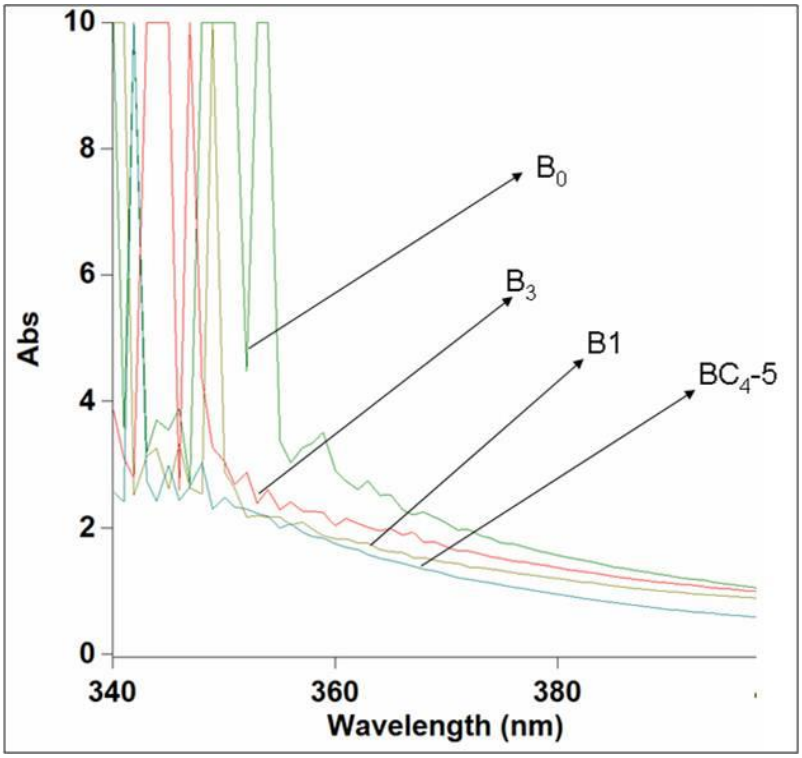


Fig.4.11.2. Absorption spectra of sardine oil before and after bleaching showing the comparison between B_0, B_1, B_3 & BC_{4E}

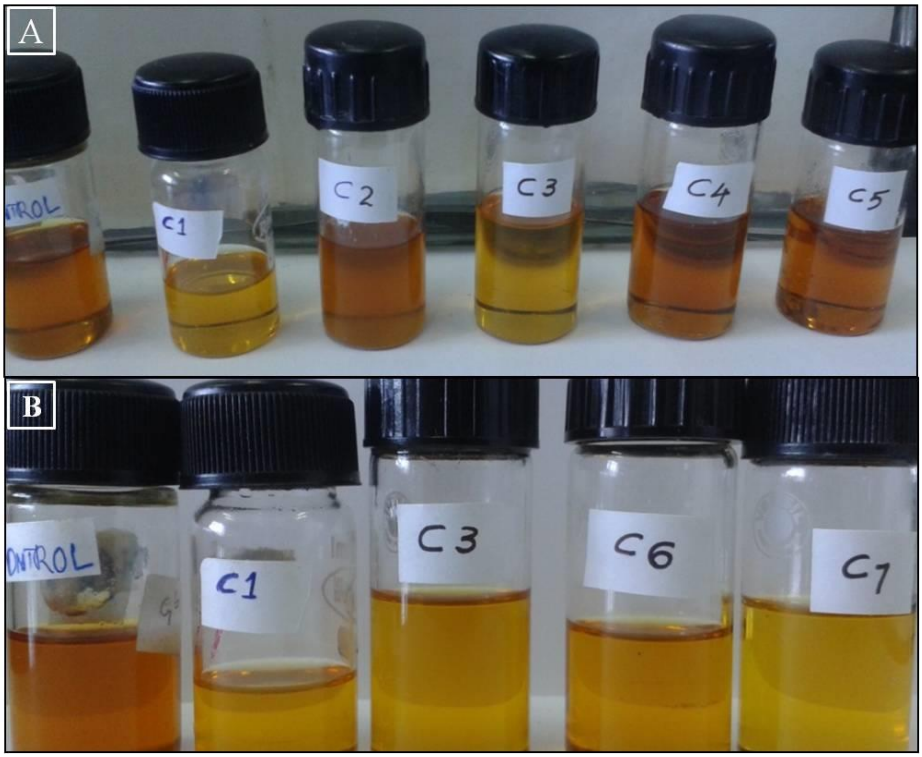


Fig.4.12. Photographs of oil bleached with (**A**) Control = Neutralized Oil, C1= B_1, C2 = B_2, C3= B_3, C4 = B_4 and C5 = B_5; (**B**) Control = Neutralized Oil, C1 = BC_4, C3 = BC_{4B}, C6=BC_{4H} and C7=BC_{4E}

4.2.2.3. Deodorization of the Bleached Oil

4.2.2.3.1. Characteristics of the Deodorized Oil

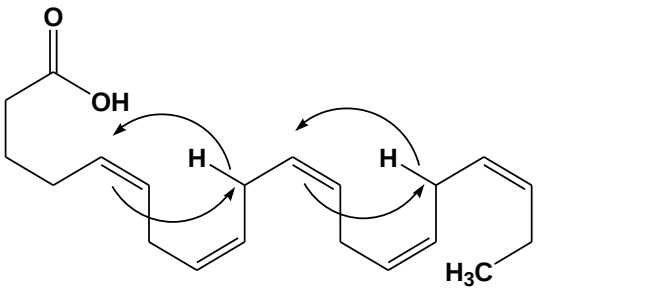
The bleached oil (BC_{4E}) on vacuum distillation for 1 h yielded 97.6 g of the deodorized oil with 73 % yield (w/w) with respect to E_6. A reduction in FFAs after the deodorisation step (DO_4) was observed (4.08 %) and this might be due to the vaporization of odoriferous compounds and free fatty acids during the deodorisation process (Ceriane & Meirelless 2007). The deodorisation step reduced FFA content by up to 47 % to afford an acceptable FFA level (i.e., between 1.8 - 3.5%) (Shativel *et al*., 2003) for the refined oil. A reduction in P.V., pA.V and TOTOX values was observed (Table 4.8) after the deodorisation process. It is significant to note that P.V. recorded below the threshold level of acceptance for human consumption (8 $meqO_2$/kg oil) (Boran *et al*., 2006), which indicated the oxidative quality index of the refined sardine oil. Thus it is evident that the conditions used for the deodorisation in the present study did not induce oxidative deterioration, which might involve the formation of ring cyclization of fatty acids and formation of various forms of cyclic fatty acid analogues. An example of the formation of the cyclic fatty acid product 5-(2-((6-(prop-1-enyl) cyclohex-3-enyl) methyl) cyclopent-3-enyl) pentatonic acid via the intermediate 5-(2-((2-but-1-enyl) cyclopent-3-enyl) methyl) cyclopent-3-enyl) pentanoic acid from eicosapentaenoic acid has been proposed under Fig. 4.13. Hydrogenated cyclic fatty acid monomers (CFAMs) formed from EPA and DHA have been characterized in an earlier study and these compounds were demonstrated to create unsavory odor in the oil (Berdeaux *et al*., 2007). Free radical-induced oxidation of DHA-containing lipids were reported to generate a seven-carbon hydroxylated acid with an aldehyde group (4-hydroxy-7oxohept-5-enoic acid), which can react with proteins generating carboxyethyl pyrrole adducts (Gu *et al*., 2003). The reduction in the content of TBARS in the deodorized oil in relation to E_6 was found to be about 40 percent. At higher temperatures isomerization and migration of double bonds is a well known phenomenon leading to the formation of extended conjugation and absorption of larger amounts of blue light, thereby resulting in the increase of orange and brown colors in oil. However, a lesser increment in L*, a*, b* values for the deodorized oil

(DO_4) than in bleached oil confirmed the absence of formation of conjugation in the fatty acid double bonds due to heating.

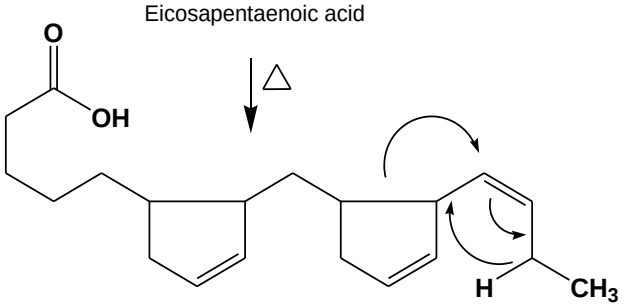
Table 4.8 Yield, P.V., pA.V., TOTOX and TBARS values of the deodorized oil

	Bleached Oil	Deodorized Oil
Yield (%)		97.62
P.V	3.65	4.58
pA.V	9.4	9.98
TOTOX	16.7	19.14
TBARS	3.62	3.69

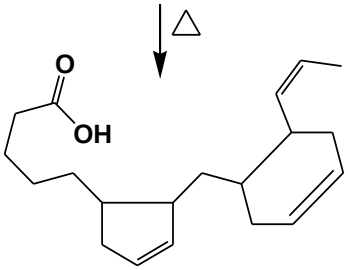
Bleached Oil - BC_{4E} obtained by bleaching with 3% concentration of the combination of activated charcoal:Fuller's earth, (1.25:3.75) at 40 min stirring at 60 ^{0}C (BC_{4E}).
Deodorized Oil - Oil deodorized by mixing bleached oil with aqueous acid solution mixture (0.25N acetic acid) and heated to 100 ^{o}C under vacuum for 60 min (ie. DO_4 *Section 4.1.3.3*).



Eicosapentaenoic acid



5-(2-((2-but-1-enyl)cyclopent-3-enyl)methyl)cyclopent-3-enyl)pentanoic acid



5-(2-((6-(prop-1-enyl)cyclohex-3-enyl)methyl)cyclopent-3-enyl)pentanoic acid

Fig. 4.13. Mechanism showing the formation of a cyclic fatty acid analogue (5-(2-((6-(prop-1-enyl)cyclohex-3-enyl) methyl)cyclopent-3-enyl)pentanoic acid) from EPA resulting in the formation of unpleasant odor

4.2.2.3.2. Fatty Acid Composition of the Deodorized Oil

The contents of ∑SFA, ∑MUFA and ∑PUFA did not show significant difference ($p > 0.05$) between the CSO and deodorized or refined (DO_4 or RO) sardine oil (Table 4.9). An insignificant reduction ($p>0.05$) in *n*-3 PUFA (percent decrease of 2.4 %) and DHA (percent decrease of 5.2 %) contents was observed in DO_4 as compared with CSO. A study by Sathivel et al. (2003) reported an approximate 19 % reduction in the *n*-3 PUFAs of catfish viscera after the oil refining processes including degumming, neutralizing, bleaching, and deodorization. The share of EPA and DHA in the refined fish oil was 20 %, which was greater than earlier cited cited (Luzia *et al*., 2003) for crude oils of five commercially important species of Brazilian fish mainly, sardine, croaker, tilapia, curimbata and shrimps (11.6 - 13.4%). The increase in the content of *trans* fatty acids was statistically insignificant ($p > 0.05$), after the process of deodorization, which might be due to the exposure of fatty acid at the high temperature used during the deodorization step (Berdeaux *et al*., 2007; Fournier *et al*., 2007).. The contents of *trans* fatty acids in the refined oil obtained in this study recorded below the threshold limit (1.0 %) as prescribed by EU guidelines (Ceriane & Meirelless, 2007) An increase in *n*-3/*n*-6 PUFA was observed for deodorized oil in relation to E_6, which show the superior nutritional qualities of the refined sardine oil. An increase in the human dietary *n*-3/*n*-6 fatty acid ratio observed for deodorized oil showed their ability to prevent coronary heart disease by reducing plasma lipids, and to reduce cancer risk (Vidal *et al*., 2012). In this work, the deodorized oil with superior qualities was obtained with the utilization of a lesser process time as compared to the conditions utilized by Fournier et al. (2007).

Table 4.9 Fatty acid profile of the deodorized oil compared with bleached oil

Fatty acids	Bleached Oil	Deodorized Oil
Saturated		
14:0	11.62±1.16	12.91±1.29
15:0	0.66±0.07	0.68±0.07
16:0	20.11±1.02	21.8±1
17:0	0.64±0.06	0.73±0.07
18:0	4.29±0.43	4.31±0.43
20:0	0.32±0.03	0.34±0.03

22:0	0.5±0.05	0.05±0.01
24:0	0.07±0.01	0.03±0
∑SFA	38.21±2.83	40.86±1.9
Monounsaturated		
14:1*n*-7	0.06±0.01	0.13±0.01
15:1*n*-7	0.01±0	0.16±0.02
16:1*n*-7 *trans*	0.01±0	0.04±0
16:1*n*-7	12.44±1.24	13.22±1.32
18:1*n*-9 *trans*	0.02±0	0.03±0
18:1*n*-9	11.54±0.14	9.53±0.95
20:1*n*-9	0.64±0.06	0.6±0.06
22:1*n*-9	3.23±0.32	2.94±0.29
24:1*n*-9	0.54±0.05	0.16±0.02
∑MUFA	28.49±0.86	26.79±2.68
Polyunsaturated		
18:2*n*-6 *trans*	0.02±0	0.03±0
18:2*n*-6	1.87±0.19	1.89±0.19
18:3*n*-6	0.56±0.06	0.41±0.04
18:3*n*-3	0.29±0.03	0.66±0.07
20:2*n*-6	1.26±0.13	1.25±0.13
20:3*n*-6	0.06±0.01	0.13±0
20:4*n*-6	0.76±0.08	0.49±0.06
20:5*n*-3 EPA	15.25±1.24	14.67±1.47
22:5*n*-3	0.75±0.08	0.53±0.05
22:6*n*-3 DHA	5.81±0.58	5.71±0.58
∑PUFA	26.63±2.69	25.78±2.58
EPA+DHA	21.06±2.11	20.38±2.04
∑*n*-3PUFA	22.1±2.11	21.57±2.16
∑n-6PUFA	4.51±0.45	4.17±0.41
∑*n*-3/∑*n*-6	4.9±0.49	5.17±0.54
∑*n*-6/∑*n*-3	0.2±0.01	0.19±0.02
∑PUFA/∑SFA	0.7±0.08	0.63±0.05
∑TRANS	0.05±0.01	0.1±0.01

Data are expressed as mean ± standard deviation of three replicates. Statistical difference ($p<0.05$) was not observed. Other notations are as represented in Table 4.3.

4.2.2.3.3. GC/MS Analysis of the Volatile Components during Deodorization

The traces of volatile odor compounds during the deodorization process with acetic acid treatment after 5 min (DO_1), 15 min (DO_2), 30 min (DO_3) and 60 min (DO_4) min of distillation were shown in Table 4.10. Fig. 4.14.1 - 4.14.4 showed the total ion chromatogram of the volatile components of the deodorized oil used in this study. Table 4.10 listed the volatile components identified in the fish oil during the deodorization process with acetic acid treatment after 5, 15, 30, and 60 min of

distillation. The distillate obtained after 5 min (as in DO_1) showed siloxane derivatives such as octamethyl cyclotetrasiloxane, decamethyl cyclopentasiloxane, dodecamethyl cyclohexasiloxane, tetradecamethyl cycloheptasiloxane, hexadecamethyl cycloocatasiloxane (Table 4.10, Fig. 4.14.1). The appearance of these siloxane derivatives could be attributed to the reaction between the stationary phases of the GC reverse phase column with the eluted components. GC-MS spectra of the distillate obtained after 15 min (DO_2) showed the presence of volatiles, mainly alkanes/alkenes (dodecane R_t 9.19 min, tetradecene R_t 11.81 min, tetradecane R_t 11.91 min, pentadecane R_t 13.16 min). The dodecane was earlier identified in menhaden fish oil, whitefish, and tuna oil (Hsieh *et al*., 1989; Josephson *et al*., 1991; Crawford *et al.,* 1976) whereas pentadecane was identified in the menhaden fish oil and salmon oil (Hsieh *et al.,* 1989; Josephson *et al*., 1983). Pentadecane was the major *n*-alkane found in oil sardines and horse mackerel oil (Moffat *et al*., 1995). GC-MS spectra of the distillate after 30 min (DO_3) showed four abundant aldehydic volatile compounds, *viz*., (E, Z)-2, 6-nonadienal, (E, E)- isomers of 2, 4-nonadienal and 2, 4-decadienal, and an alcohol, E-2-octadecadecen-1-ol (Table 4.10). Aldehydes play an important role in many food products and are responsible for a wide range of oxidized flavor (Vejaphan *et al*., 1988) Milo and Grosch (1996) demonstrated that (E, Z)-2, 6-nonadienal and (E, E)-2, 4-decadienal were the key aroma compounds of raw and boiled salmon. GC-MS spectra of the distillate obtained after 60 min (DO_4) demonstrated the presence of two prominent aldehydes *viz*., (E, E) - isomers of 2, 4-heptadienal and 2, 4-decadienal (Fig.4.14.4, 5 & 6). Rahimabadi et al. (2011) recorded (E, Z)-2, 6-nonadienal and (E, E)-2, 4-heptadienal on the distillation of raw Narrow-barred Spanish mackerel (*Scomberomorous commerson*). The volatiles such as (E, Z)-2, 6-nonadienal and (E, E) - 2, 4-decadienal were separated earlier from the cod liver oil by means of deodorization at 200 ^{o}C for 2 h (Karahadian & Lindsay 1990). (E, Z)-2, 6-Nonadienal was identified as the compound contributing to the green and melon flavors encountered in fish oils (Karahadian & Lindsay 1989). Furthermore, (E, E)-2,4-heptadienal was reported as strong fat odor and fish smell inducing compound in the oil (Roh et al., 2006). The presence of these unsaturated volatile aldehydes in the fish oil was apparently due to the greater contents of PUFAs. A schematic representation of the peroxidation of polyunsaturated fatty acids (eicosapentaenoic and linoleic acids have been taken as a model systems) leading to the formation of aldehydes was shown in Fig.4.14.5 & 6.

It is challenging to characterize the long-chain fatty aldehydes (in free forms) within biological matrices apparently due to their greater reactive properties. These highly unstable compounds react with nucleophiles in the matrix leading to their rapid metabolism. Of the fatty aldehydes, 2, 4-decadienal (particularly from the n-6 fatty acids) and hepta-2, 4-dienal (from *n*-3 fatty acids) are of greater importance due to their contribution to the characteristic unpleasant odor of the oil.

The hydroperoxides formed by free radical induced autoxidation process of *n*-3 polyunsaturated acids are highly unstable, and undergo prompt β-scission to form the carbonyl compounds such as hepta-2, 4-dienal. For example, 14-hydroperoxyicosa-5,8,11,15,17-pentaenoic acid has been demonstrated to be formed from EPA (20:5*n*-3) by free radical induced formation of conjugated double bond, which thereafter form hepta-2,4-dienal as predominant reaction product. Electron ionization (EI)-induced mass spectrometry of hepta-2,4-dienal with a conjugated diene system yields characteristic molecular ion at *m/e* [M]+ 110, and the fragment ions at *m/e* 114 (heptanal), *m/e* 70 (pent-1-ene), and *m/e* 58 (prop-1-en-2-ol) due to reduction of molecular ion followed by intramolecular McLafferty type of rearrangement. The β-cleavage of heptanal (*m/e* 114) with the gain of γ-hydrogen atom led to the formation of pent-1-ene and prop-1-en-2-ol. Hepta-2, 4-dienal generates prominent heterocyclic product ions with *m/e* 82 (2H-pyran), *m/e* 84 (3, 6-dihydro-2H-pyran), and *m/e* 70 (2, 5-dihydrofuran) (Fig. 4.14.5) by intramolecular rearrangement of the parent molecular ion.

The diene *n*-6 polyunsaturated fatty acids have been shown to be good precursor in some defined oxidative conditions to form deca 2, 4-dienal along with other reaction products. To rationalize the formation of aldehydic products, as is the case for 2, 4-decadienal, linoleic acid (18:2*n*-6) has been taken as an example. This *n*-6 dienoic fatty acid may undergo oxidation via 9-hydroperoxyoctadeca-10, 12-dienoic acid forming the fatty acid aldehyde with a conjugated diene system (2, 4-decadienal) as the major product. The conjugated unsaturated aldehydes may undergo changes by autoxidation producing shorter chain aldehydes and other volatile compounds (data not shown). EI-induced mass spectrometry of deca 2,4-

dienal yields characteristic molecular ion at *m/e* [M]+ 152, and the fragment ions at *m/e* 150 (4-ethyl-3a,4,5,6-tetrahydrobenzofuran) due to intramolecular rearrangement, and at *m/e* 122 (3a,4,5,6-tetrahydrobenzofuran) due to the loss of ethyl ion from the former. In particular, deca 2, 4-dienal generates prominent heterocyclic product ions with *m/e* 82 (2H-pyran), *m/e* 84 (3,6-dihydro-2H-pyran), and *m/e* 70 (2,5-dihydrofuran), as demonstrated for hepta-2, 4-dienal (Fig. 4.14.6).

Other volatile decomposition products of hydroperoxides reported in severely oxidized fish oil were alkatrienals, mainly 2,4,7-decatrienal (P.V. above 70 meq/kg) (Ke *et al*., 1975) However, in the present study no trace of alkatrienal in the distillate was observed. Thus, a distillation period of one hour could optimally able to remove the major volatile components from the sardine oil.

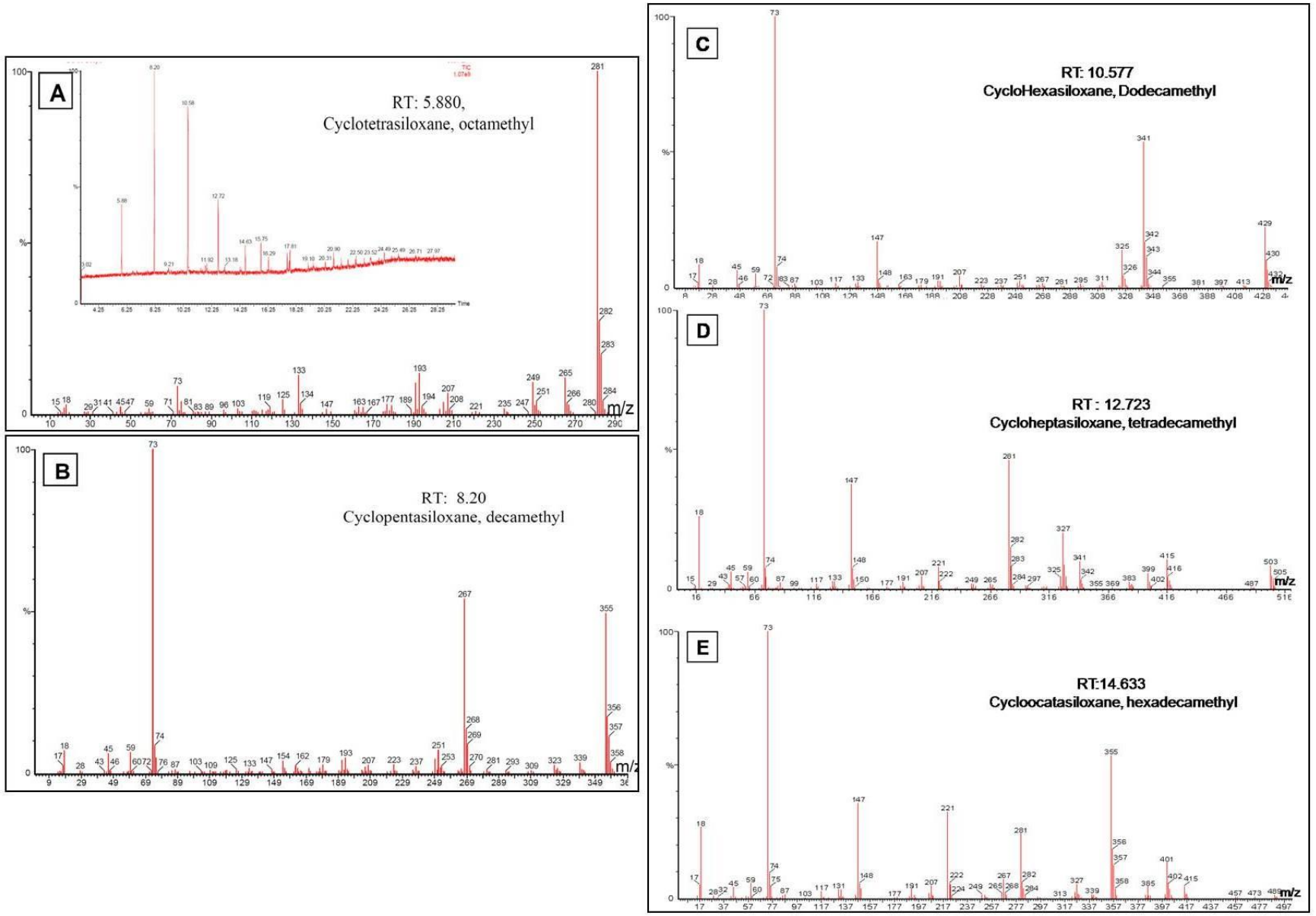


Fig.4.14.1. GC/MS of DO1 (A) Cyclotetrasiloxane, (B) Cyclopentasiloxane, (C) CycloHexasiloxane, Dodecamethyl (D) Cycloheptasiloxane, tetradecamethyl, (E) Cycloocatasiloxane, hexadecamethyl

Fig.4
Cyclo

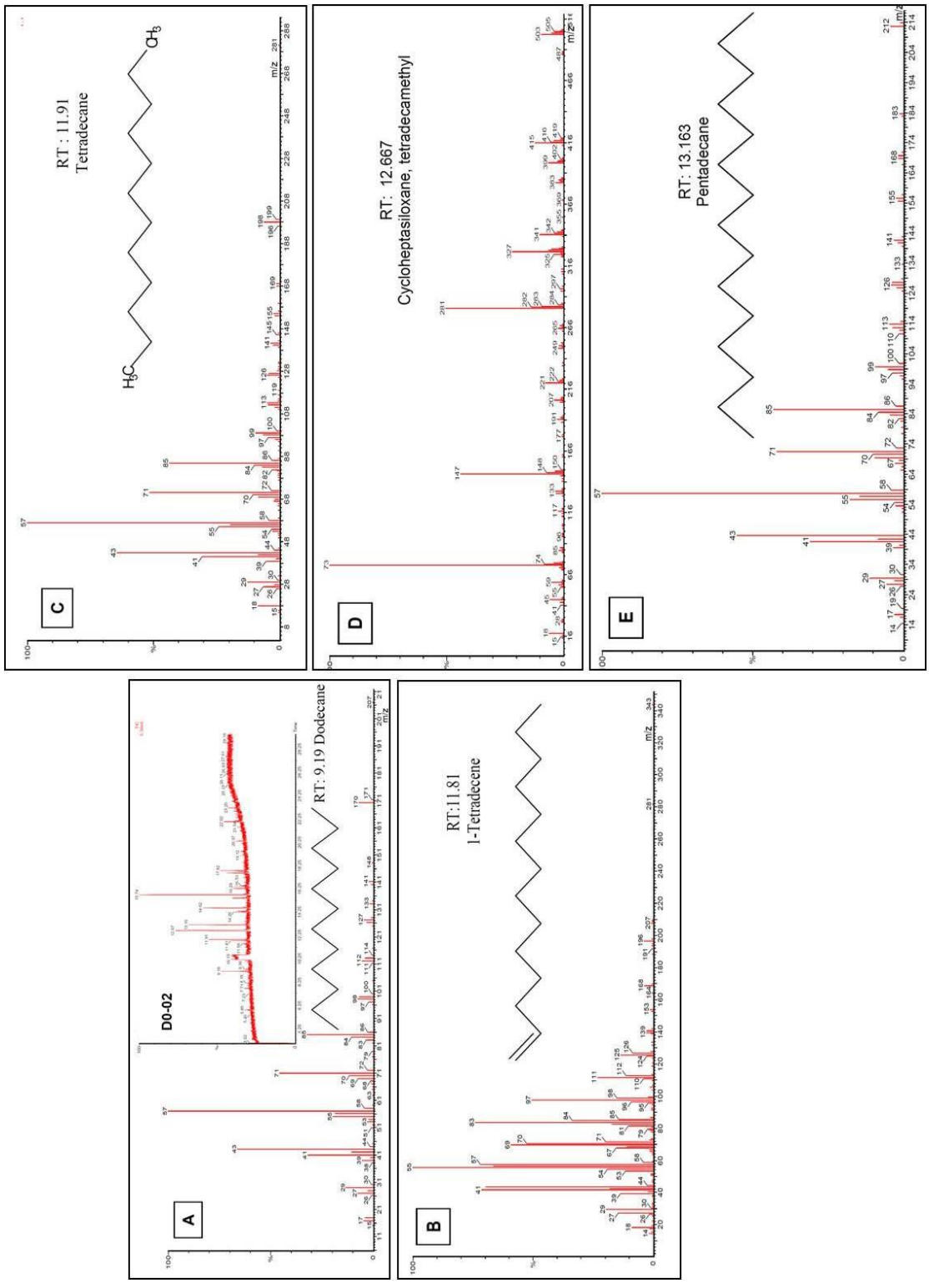


Fig. 4.14.2. GC/MS of DO_2 (A) Dodecane, (B) 1-Tetradecene, (C) Tetradecane, (D) Cycloheptasiloxane, tetradecamethyl, (E) Pentadecane

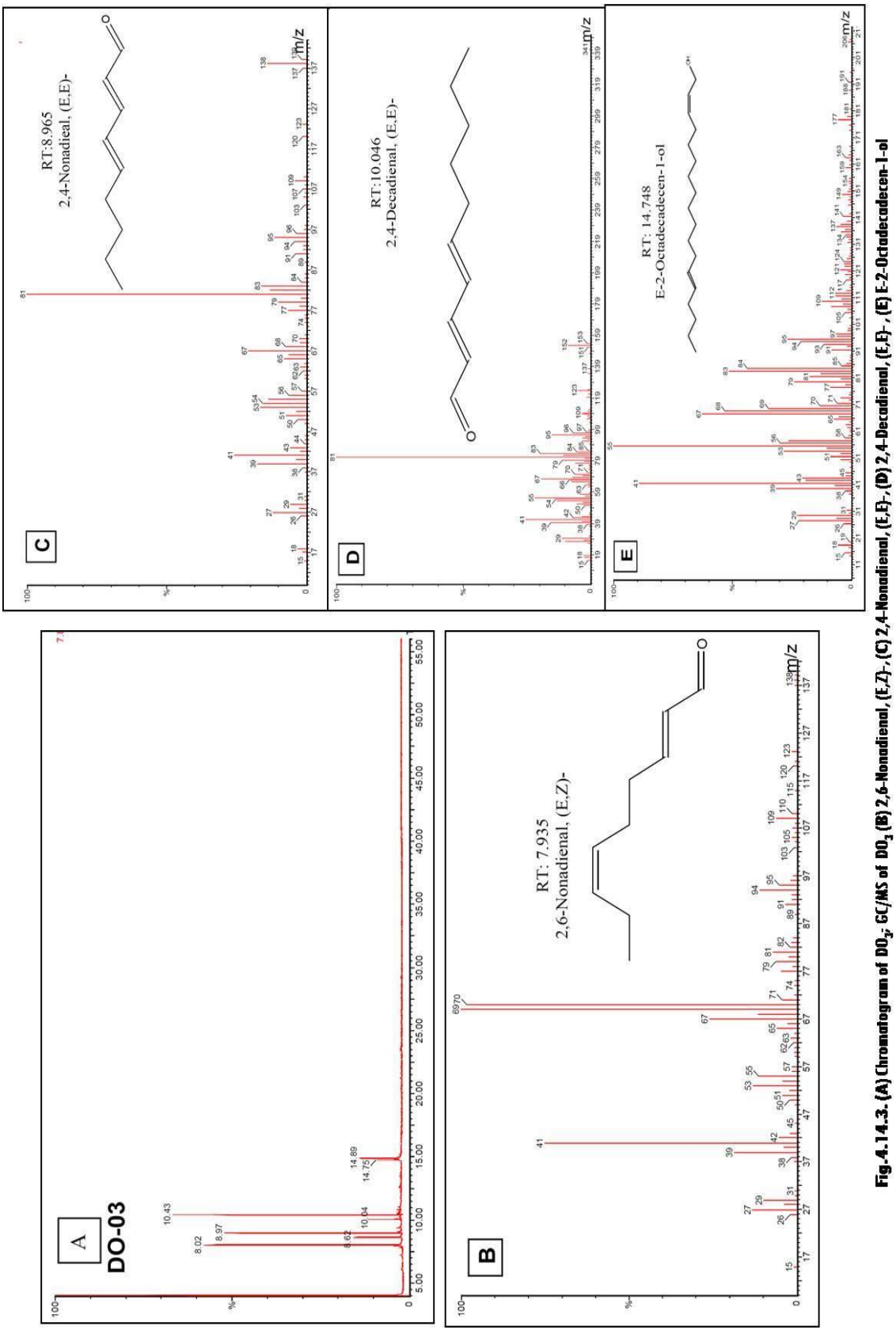


Fig.4.14.3. (A) Chromatogram of DO_3; GC/MS of DO_3 (B) 2,6-Nonadienal, (E,Z)-, (C) 2,4-Nonadienal, (E,E)-, (D) 2,4-Decadienal, (E,E)- , (E) E-2-Octadecadecen-1-ol

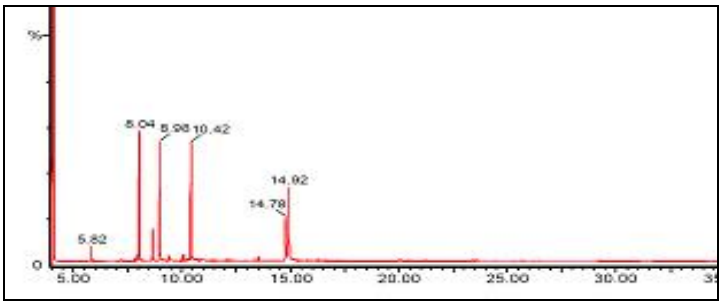


Fig.4.14.4. Chromatogram of DO_4

Table 4.10 Volatile compounds obtained during the deodorization process of the sardine oil.

Peak number	R_t (min)	Compounds	Base peak*	Molecular Weight [M^+]
DO_1				
1	5.880	Octamethyl cyclotetrasiloxane**	281	284
2	8.201	Decamethyl cyclopentasiloxane**	73	358
3	10.577	Dodecamethyl cyclohexasiloxane**	73	432
4	12.723	Tetradecamethyl cycloheptasiloxane**	73	505
5	14.633	Hexadecamethyl cyclooctasiloxane**	73	489
DO_2				
1	9.19	Dodecane	57	207
2	11.81	Tetradecene	55	343
3	11.91	Tetradecane	57	281
4	12.667	Tetradecamethyl cycloheptasiloxane**	73	505
5	13.163	Pentadecane	57	212
DO_3				
1	8.015	(E,Z)-2,6-Nonadienal	41	138
2	8.965	(E,E)-2,4-Nonadienal	81	139
3	10.046	(E,E)-2,4-Decadienal	81	341
4	14.748	E-2-Octadecadecen-1-ol	55	206
DO_4				
1	5.819	(E,E)-2,4-Heptadienal	81	111
2	10.416	(E,E)-2,4-Decadienal	81	153

* Base peak in GC/MS spectra refers to the *m/e* peak with 100% intensity.

** The siloxane derivatives of fatty acids were formed by reaction between the GLC column stationary phase and the fatty acid reaction products.

DO_1, DO_2, DO_3 and DO_4 – Distillates obtained at 5, 15, 30 and 60 min, respectively during the deodorization process of the sardine oil. The prefixes (E)-(Z)- denote *trans*- and *cis*-isomers, respectively.

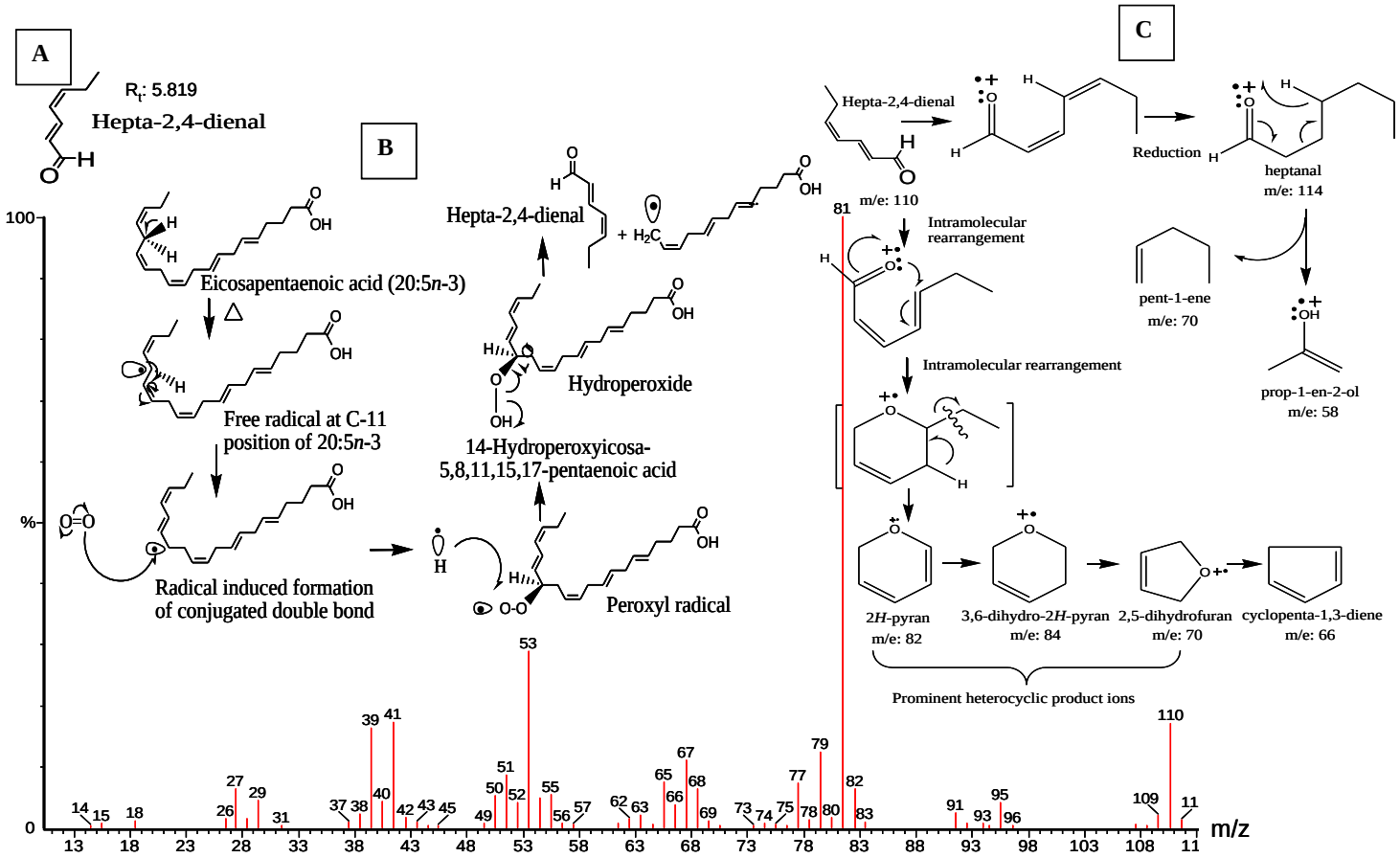


Fig.4.14.5. (**A**) GC/MS spectrum of 2, 4-heptadienal; (**B**) free radical induced formation of 2, 4-heptadienal from *n*-3 polyunsaturated fatty acids (EPA, 20:5*n*-3); (**C**) Proposed GC/MS fragmentation pathways for generation of fragment ions during EI-MS analysis of 2, 4-heptadienal from DO_4.

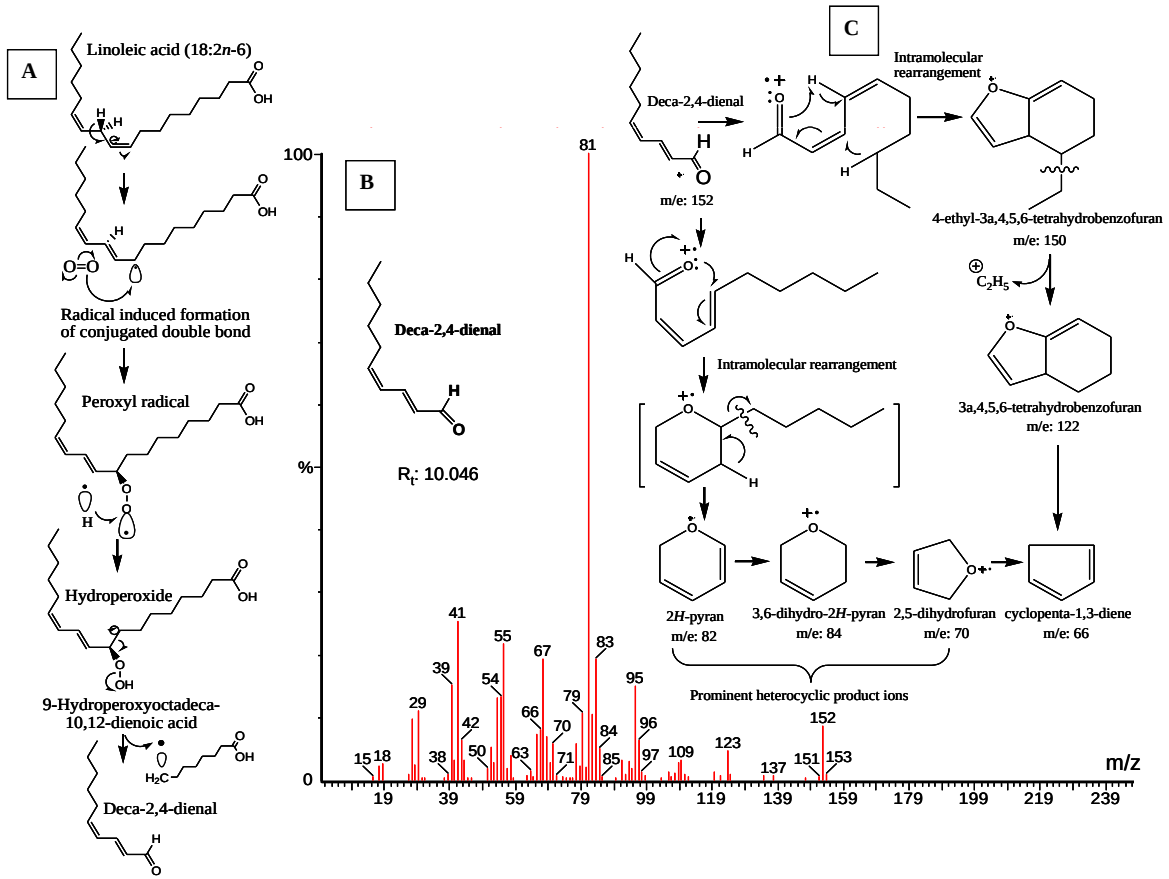


Fig. 4.14.6. (**A**) Free radical induced formation of 2, 4-decadienal from *n*-6 polyunsaturated fatty acids (linoleic acid, 18:2*n*-6); (**B**) GC/MS spectrum of 2, 4-decadienal; (**C**) proposed GC/MS fragmentation pathways for generation of fragment ions during EI-MS analysis of 2, 4-decadienal from DO_4.

4.3. Conclusions

The effect of different extraction methods on the quality parameters of the crude sardine oil was evaluated. The extraction of the oil from *Sardinella longiceps* using the process of cooking followed by pressing have been demonstrated to be important to obtain the good quality oil with respect to the lipid yield and broad spectrum oil characteristics. The fatty acid composition was found to be comparable with the conventional Bligh and Dyer method with respect to polyunsaturated fatty acids (> 26 %), EPA (> 15 %) and DHA (> 4.5%) contents. The oil obtained by optimum methodology of extraction was further characterized by FTIR, NMR and GCMS spectral analysis to understand the signature peaks and relative abundance of different fatty acids in a faster and more reliable manner. The crude oil obtained from *Sardinella longiceps* was refined by the step-wise process of degumming, bleaching and deodorization. The effective degumming agent was found to be 1% phosphoric acid, which could able to keep the P.V, pA.V, TOTOX, TBARS below threshold limits. The utilities of NMR-based proton signal characteristics as new analytical tools to understand the signature peaks and relative abundance of different fatty acids and monitoring the refining process of fish oil have been demonstrated. The region between δ 4-4.5 ppm in the ^{1}H-NMR spectra was taken into account to explain the bleaching process of the oil, and the characteristic signals at δ1.8 – 3.0, δ 3.0 – 4.5, and δ 4.5 – 6.5 ppm were considered as the indicator regions to explain the variability of the oil qualities under different treatment combinations during the degumming process. Significant reduction in the contents of hydrocarbon functionalities (as shown by the decrease in proton integral in the characteristic ^{1}H-NMR region) was demonstrated by using 1% H_3PO_4 during the course of degumming process. A combination of activated charcoal and Fuller's Earth (1.25:3.75, 3% w/w, contact time of 60 min) was found to be efficient in bleaching the sardine oil, and to eliminate the colored impurities. This study also demonstrated that unfavorable odor-causing components particularly low molecular weight carbonyl compounds and alcohols could successfully be removed by distillation under vacuum with 0.25N acetic acid (100 ^{o}C). The four stage refinement process of

sardine oil resulting in a significant improvement in nutritional values and fish oil characteristics have been demonstrated. This study demonstrated that the degumming process by using phosphoric acid (1%) followed by bleaching by using the adsorbent combination of activated charcoal- Fuller's Earth (1.25:3.75, w/w, 3%), and deodorization by using distillation for one hour could be effective to afford a refined sardine oil with superior quality.

Chapter 5

PREPARATION OF PURIFIED ESSENTIAL FATTY ACIDS

Contents

Background

The polyunsaturated fatty acids (PUFAs) are currently in demand in the pure form in nutraceutical and aqua feed formulations, and are being actively studied to understand their potential roles in human health. The technologies available for purifying individual PUFAs and PUFA concentrates from fish oil are based on the differences in physicochemical properties associated with the number of double bonds in the molecule or the chain length. However, most of the existing chemical methods for purifying individual PUFAs and PUFA concentrates are non-selective for different fatty acids. The substrate specificity of lipases (triacylglycerol acyl hydrolases) has also been utilized for the enhancement of PUFA content in fish oils by several groups (Liu *et al.*, 2007).

The present chapter highlights the methodologies for purification of PUFAs from refined sardine oil with the goal to get a PUFA with high eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3). The objective in this chapter is to purify the *n*-3 PUFAs, particularly EPA and DHA from sardine oil by three major steps: (1) saponification of fish oil to derive free fatty acids, (2) winterization of the free fatty acids at different temperatures (-20 and 4 ^{o}C) using the solvents ethyl acetate, acetone and their different combinations (Me_2O: EtOAc – 7:3, 1:1 & 3:7 % v/v), (3) concentration of PUFAs from the winterized fatty acids by urea complexation at three different temperatures (4, 10, and -20 °C) and urea: oil ratios (2:1, 3:1, and 4:1 w/w), contact time (12, 24 and 36 h) and (4) purification of the concentrated *n*-3 fatty acids which converted to its methyl esters by normal pressure argentated alumina/silica column chromatography. The present chapter is also directed to prepare *n*-3 PUFA concentrates by lipase-catalyzed purification of the refined oil, and further concentrating the target PUFAs by the optimum methods of urea complexation and argentated column chromatography. Structure–activity relationship analyses, to correlate the lipase activity

with physico-chemical properties of different classes of fatty acids were used to elucidate the structural descriptors of fatty acids (hydrophobic and steric) controlling lipolytic activity. The characteristics of PUFAs obtained at different stages of purification were also studied. A schematic diagram showing the overall process of purification is shown in Fig. 5.1.

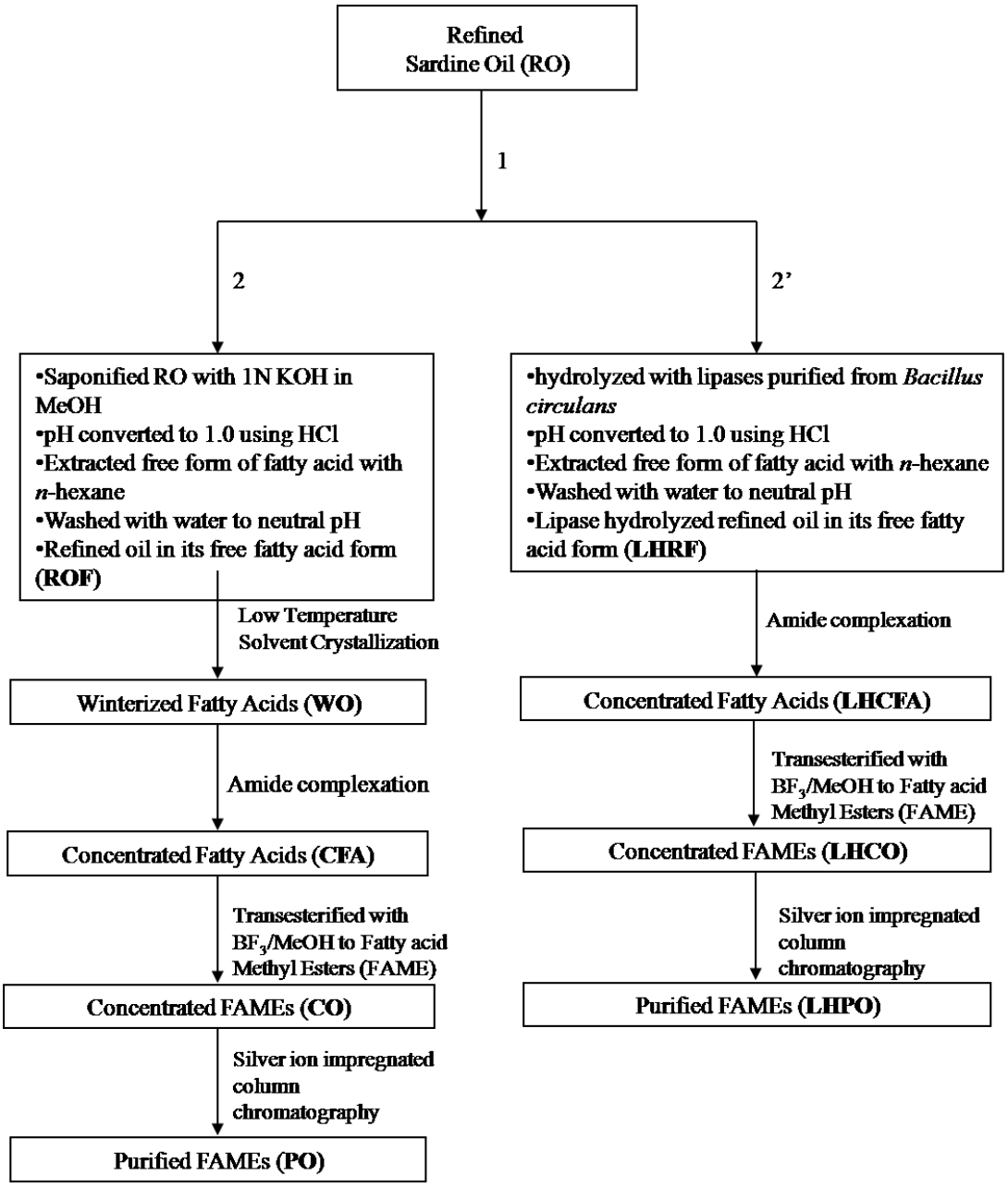


Fig. 5.1. Schematic diagram showing the winterization, concentration and purification of refined sardine oil to obtain purified methyl esters

5.1. Materials and Methods

5.1.1. Chemicals, Reagents and Instrumentation

Neutral alumina (0.06 - 0.2 mm, MW 101.96; column chromatography grade, Sephadex G-100, Tris–HCl, $NH_4(SO_4)_2$, CH_3COOH, $CaCl_2$, NaCl, $AgNO_3$, 4-nitrophenyl palmitate (4-NPP), bovine serum albumin (BSA), carbonic anhydrase, soybean trypsin inhibitor, ovalbumin, lysozyme *β-phosphorylase*, PIPES–NaOH buffer (5 ml, 0.15 M, pH 7.0), Triton X-100, 2,7-dichlorofluorescein, rhodamine B-triolein agar, Coomassie Brilliant Blue R-250, BF_3 in MeOH (14 %, w/w), glacial acetic acid (gl.AcOH) etc were used in this study. All these reagents were of analytical grade and purchased from E-Merck (Darmstadt, Germany) or HiMedia (Mumbai, India). Low-range molecular mass markers (14.4 - 97.4 kDa) were purchased from Bio-Rad Laboratories, Hercules CA. All the solvents *viz*., CH_3OH, diethyl ether, $CHCl_3$, dichloromethane (DCM), *n*-hexane, Me_2O were redistilled in an all-glass system and degassed with N_2 prior to use. Doubly distilled water was used throughout this work.

The instrumentation used such as Gas-liquid chromatography (GLC), FTIR, ^{1}HNMR, ^{13}C-NMR, UV-VIS spectrophotometer (Varian Cary 50 Conc., USA), GC-MS, LC-MS, UV-VIS spectrophotometer, lyophilizer, rotary vacuum evaporator are as explained in the previous chapters (*Chapter 3 & 4*).

5.1.2. Saponification and Extraction of Free Fatty Acids from Refined Sardine Oil

About 100 g refined sardine oil (RO; *Chapter 4*) was saponified by refluxing with 500 ml of alkaline KOH solution (1N in MeOH) under continuous stirring for 45 min in an inert atmosphere of N_2 following the procedure adopted by Haagsma (Haagsma *et al.,* 1982) with modifications. The hydrolyzed mixture was diluted with water (500 ml), and the unsaponifiable matter was extracted successively with diethyl ether (200 ml x 2) followed by *n*-hexane (200 ml x 2) and were discarded. The pH value of the lower hydroalcoholic phase containing the saponified fatty acids was adjusted to 1.0 with HCl-H_2O (1:1 v/v, 150 ml), and the resultant free fatty acids

were recovered by extraction with *n*-hexane (200 ml x 4), washed with water to neutral pH, and dried over anhydrous Na_2SO_4. The residual solids were removed with a Buchner funnel under suction, and the solvent was evaporated to recover the resultant refined oil in its free fatty acid form (ROF).

5.1.3. Preparation of Purified Essential Polyunsaturated Fatty Acids using Physical and Chemical Techniques

The essential fatty acids in the above obtained free fatty acid form of refined sardine oil (ROF, *Section 5.1.2*) was purified by employing winterization (physical), urea complexation (chemical) and argentation chromatographic (chemical) techniques.

5.1.3.1. Winterization of Refined Sardine Oil

The refined sardine oil in its FFA form (ROF) was treated with different solvent/solvent systems at different crystallization temperatures as depicted in Table 5.1. The solvents Me_2O and EtOAc individually and their three different proportions of were used as solvents for RO winterization (70, 50 and 30%, v/v) with oil: solvent ratio of 1:10. The crystallization temperature was initially (within 1 - 3 h) reduced moderately. Thereafter winterization process was carried out at different temperatures (-20 and 4ºC) for a contact time of 12 h. The liquid phase was separated, filtered through Buchner funnel, dried over anhydrous Na_2SO_4, concentrated in a vacuum rotary evaporator at 40-50 ºC to obtain the winterized sardine fatty acids (WO). The optimum winterization method was selected by analyzing the fatty acid composition of both the concentrated liquid phase (non-crystallized portion) and solid phase (crystallized portion). The percent recovery, P.V., pA.V., TOTOX, and TBARS values (*protocols as explained in Chapter 4; Section 4.1.4*) of the winterized fatty acids was also evaluated. The fatty acid composition of WO was analyzed by converting to their methyl esters as detailed earlier. The optimum winterization method selected was further evaluated by UV-VIS scanning, FTIR and ^{1}H-NMR analyses.

Table 5.1. Various winterization methodologies (RW_{1-10}) employed for refined sardine oil (RO) at different temperatures and solvent/solvent systems

Codes	Solvent or solvent systems used	Crystallization temperature (°C)
RW_1	EtOAc	-20
RW_2	Me_2O	-20
RW_3	Me_2O: EtOAc (7:3)	-20
RW_4	Me_2O: EtOAc (1:1)	-20
RW_5	Me_2O: EtOAc (3:7)	-20
RW_6	EtOAc	4
RW_7	Me_2O	4
RW_8	Me_2O: EtOAc (7:3)	4
RW_9	Me_2O: EtOAc (1:1)	4
RW_{10}	Me_2O: EtOAc (3:7)	4

EtOAc - Ethyl acetate; Me_2O - Acetone

5.1.3.2. Concentration of Winterized Sardine Fatty Acids (WO)

The winterized sardine fatty acids (WO) were concentrated using amide complexation (urea-fatty acid complexation) under different experimental conditions (Table 5.2). In the first step of crystallization, urea was added to the solvent (MeOH/EtOH). The mixture was heated at elevated temperature (65 ± 5 °C) with constant stirring in different ratios (Table 5.2) until a clear, homogeneous solution was formed. The winterized sardine fatty acids were added into the resulting urea solution under different experimental conditions as detailed in Table 5.2. The solution was cooled to room temperature at a cooling rate of 0.5 °C/min and subsequently crystallized at different temperatures for different contact times (Table 5.2). After thawing, the urea crystals (urea complex fraction, UCF) were separated from the mother liquor (non-urea complex fraction, NUCF) by vacuum filtration. The filtrate containing the PUFAs was evaporated using a rotary vacuum evaporator to remove the residual solvent. The solid residue thus obtained was diluted with water (200 ml) and acidified with dilute HCl (6N, 30 ml) to pH 4-5 under stirring to remove traces of residual urea and MeOH. The liberated fatty acids after acidification with dilute HCl were extracted with different solvents (*n*-hexane, chloroform, dichloromethane), and *n*-hexane was found as the best with higher

recovery percentage of fatty acids. The fatty acids were extracted from the residue with *n*-hexane three times in a separating funnel to cause the phase separation of urea and concentrated PUFAs. The upper layer of *n*-hexane containing the PUFAs was separated and dried over anhydrous Na_2SO_4. The resulting concentrated sardine fatty acids (CO) were dissolved in MeOH and kept under a blanket of N_2 at -20 °C until use. The optimum urea complexation method for concentration was selected by analyzing the fatty acid composition of both the concentrated urea complexed and non-urea complexed fractions (UCF & NUCF, respectively). The fatty acid composition of UCF and NUCF was analyzed by converting to their methyl esters as detailed earlier. The percent recovery, P.V., pA.V., TOTOX, TBARS values (*protocols as explained in Chapter 4; Section 4.1.4*) of the non-urea complex fraction (NUCF) were also evaluated. The optimum urea complexation method selected was further evaluated by UV-VIS absorption, FTIR and ^{1}H-NMR analyses.

Table 5.2 Amide complexation methodologies (U_1-U_{15}) studied for concentration of winterized sardine fatty acids (WO) at different temperatures (4 and -20°C) and solvent/solvent systems

Codes	Urea:WO ratio	Solvent used	Crystallization temperature (°C)	Contact time (h)
U_1	0:1	EtOH	4	12
U_2	4:1	EtOH	4	12
U_3	0:1	MeOH	4	12
U_4	2:1	MeOH	4	12
U_5	3:1	MeOH	4	12
U_6	4:1	MeOH	4	12
U_7	2:1	MeOH	10	12
U_8	3:1	MeOH	10	12
U_9	4:1	MeOH	10	12
U_{10}	2:1	MeOH	-20	12
U_{11}	3:1	MeOH	-20	12
U_{12}	4:1	MeOH	-20	12
U_{13}	4:1	MeOH	RT	4
U_{14}	4:1	MeOH	4	24
U_{15}	4:1	MeOH	4	36

WO - Winterized sardine fatty acids; RT - 30-32 °C; EtOH – Ethyl alcohol; MeOH - Methanol

5.1.3.3. Acid-Catalyzed *trans*-Esterification of Concentrated Sardine Fatty acids (CFA) to Concentrated Fatty Acid Methyl Esters (CO)

About 100 g of concentrated sardine fatty acids (CFA) was *trans*-esterified to furnish concentrated sardine fatty acid methyl esters (CO) by reaction (30 min under reflux condition) with 14% BF_3 in MeOH. The reaction was carried out in a heating mantle (45 – 50^{o}C) under an inert atmosphere of N_2. The FAME thus obtained was cooled to ambient temperature and distilled water (20 ml) was added. The solution was extracted with *n*-hexane (10 ml x 6), and the upper *n*-hexane layer was removed and concentrated under an inert atmosphere of N_2. The resulting FAME concentrate was flushed with N_2 before being stored at -20 °C until further processing. The reaction was followed up by TLC using *n*-hexane:EtOAc:gl.AcOH (90:10:1) as the mobile phase.

5.1.3.4. Purification of Concentrated Fatty Acid Methyl Esters (CO) by Argentated Chromatography

The concentrated sardine methyl esters (CO) was purified by means of normal pressure liquid column chromatography with $AgNO_3$- impregnated neutral alumina or silica gel as the stationary phase (detailed in following *Sections 5.1.3.4A-D*). Evaporation of solvents from the fractions, followed by silver-ion thin-layer chromatography ($AgNO_3$/TLC, 5 cm x 20 cm) using *n*-hexane/EtOEt/gl.AcOH (80:20:0.5, v/v/v), validated the purity of the individual fatty acids. The TLC bands were stained with 2, 7 - dichlorofluorescein in MeOH (0.1%, w/v), and examined under UV-light. The optimum methodology to get maximum purity of *n*-3 fatty acids has been selected by assaying the fatty acid composition of all the eluted dried fractions. The purity of the essential fatty acids eluted with most efficient method has been further validated using UV-VIS absorption, FTIR and ^{1}H-NMR analyses. The recovered fatty acid methyl esters from column chromatography were resolved by TLC (5 cm x 20 cm), precoated with silica gel, and impregnated with $AgNO_3$. In this process silica gel G (15 g) was mixed with a 10% (w/v) solution of $AgNO_3$ (40 ml) in MeOH/water (9:1 v/v) and spread in a uniform thickness (0.25 mm). The plates were drained, air-dried, activated at 110-120 °C in dim light for 30 min, and stored in

a light-tight desiccator container. The methyl esters were applied to the plate as a narrow band. The plates were developed twice in *n*-hexane/diethyl ether/acetic acid (94:5:1 v/v/v) to separate individual bands. The bands were stained with 2, 7-dichlorofluorescein in EtOH (0.1% w/v), and examined under UV light.

5.1.3.4A. Preparation of Silver Impregnated Alumina or Silica

Silver nitrate powder ($AgNO_3$, 10 g) was added to EtOH (80% v/v, 60 ml) and dissolved by stirring for 10 min. About 50 g of neutral alumina (70–230 mesh) or silica gel (0.06–0.2 mm, 70–230 mesh ASTM; mean pore diameter of 6 nm, specific surface area of 500 m^2/g), was slurried in EtOH (95% v/v, 100 ml) and added to $AgNO_3$ solution under stirring for 2h. Ethanol was evaporated under vacuum at 60 °C, and the silver impregnated alumina/ silica was activated by heating overnight (110 ± 2 °C) in the hot air oven to prepare Ag-alumina/Ag-silica powder. This material was cooled and kept in the dark in a desiccator until use.

5.1.3.4B. Argentation Chromatography using Ag-Alumina

The slurry of Ag-alumina (5 g) in *n*-hexane (5 ml) was poured into a water-jacketed column (45 cm x 50 mm i.d.) previously half-filled with *n*-hexane. The packed height of glass chromatography column (diameter - 0.5 cm) was maintained at 9 cm. The concentrated sardine methyl ester (CO, 5g) was dissolved in *n*-hexane (5 ml) and applied on the chromatography column. The column was eluted with a sequence of organic solvents and the eluates were collected as fractions corresponding to the applied solvents as shown in Table 5.3.1 to obtain 7 fractions (FA_{1-7}). The solvent was removed from the fractionated liquid layer to obtain highly purified PUFAs.

Table 5.3.1 Column elution pattern for the purification of concentrated sardine methyl ester (CO) using Ag-alumina as adsorbent in *n*-hexane/acetone

Fractions	Eluent	Solvent ratio (v/v)
FA_1	*n*-hexane	100
FA_2	*n*-hexane:Me_2O	99:1
FA_3	*n*-hexane:Me_2O	97:3
FA_4	*n*-hexane:Me_2O	95:5
FA_5	*n*-hexane:Me_2O	92:8
FA_6	*n*-hexane:Me_2O	90:10
FA_7	Me_2O	100

Me_2O - Acetone

5.1.3.4C. Argentation Chromatography using Ag-Silica

The slurry of Ag impregnated silica (5 g) in *n*-hexane (5 ml) was poured into a water-jacketed column (45 cm x 50 mm i.d.) previously half-filled with *n*-hexane. The packed height of glass chromatography column (diameter - 0.5 cm) was maintained at 9 cm. The concentrated sardine methyl ester (CO) was dissolved in *n*-hexane (5 ml) and applied on the chromatography column. The column was eluted with sequence of *n*-hexane and Me_2O. The eluates were collected as fractions corresponding to the applied solvents as shown in Table 5.3.2A to obtain 7 fractions (FS_{1-7}). The solvent was removed from the fractionated liquid layer to obtain highly purified PUFAs.

Table 5.3.2A Column elution pattern for the purification of concentrated sardine methyl ester (CO) using Ag-silica as adsorbent in *n*-hexane/acetone

Fractions	Eluent	Solvent ratio (v/v)
FS_1	*n*-hexane	100
FS_2	*n*-hexane:Me_2O	99:1
FS_3	*n*-hexane:Me_2O	97:3
FS_4	*n*-hexane:Me_2O	95:5
FS_5	*n*-hexane:Me_2O	92:8
FS_6	*n*-hexane:Me_2O	90:10
FS_7	Me_2O	100

Me_2O - Acetone

In order to evaluate the efficacy of another solvent system the column elution of CO in Ag-silica was performed using the step gradient diethyl ether:*n*-hexane to obtain 7 fractions (FD_{1-7}) (Table 5.3.2B).

Table 5.3.2B Column elution pattern for the purification of concentrated sardine methyl ester (CO) using Ag-silica as adsorbent in diethyl ether/*n*-hexane

Fractions	Eluent	Solvent ratio (v/v)
FD_1	*n*-hexane	100
FD_2	*n*-hexane:EtOEt	90:10
FD_3	*n*-hexane:EtOEt	80:20
FD4	*n*-hexane:EtOEt	70:30
FD_5	*n*-hexane:EtOEt	60:40
FD_6	*n*-hexane:EtOEt	50:50
FD_7	EtOEt	100

EtOEt - diethyl ether

5.1.3.4D. Stirring with Ag-silica

The concentrated sardine methyl ester (CO) was dissolved in *n*-hexane (5 ml) and 2 g of Ag-silica was added and the content was stirred (60 ^{o}C) with *n*-hexane, *n*-hexane:Me_2O (1:1) and Me_2O in a sequential order. The solution of CO containing Ag-silica was filtered *in vacuo* separately and concentrated to furnish three fractions, FT_1, FT_2 and FT_3.

5.1.4. Preparation of Purified Essential Fatty Acids using Biotechnological, Physical and Chemical Techniques

The refined oil of *S. longiceps* (RO; *Chapter 4*) was purified by employing biotechnological (enzymatic hydrolysis) followed by physical (winterization) and chemical (urea complexation and argentated chromatography) techniques.

5.1.4.1. Isolation, Production, Purification and Molecular Weight Determination of Lipase from *Bacillus circulans*

Amongst several epiphytic bacteria isolated from the brown seaweed *Turbinaria conoides*, one Bacillus sp. (*Bacillus circulans*) was used for production of lipase due to its higher lipolytic potential. The crude lipase thus obtained was purified to homogeneity by ammonium sulphate precipitation, sephadex G-100 gel filtration, and amberlite IRA-410 (Cl^- form) anion-exchange chromatography. The molecular weight of the lipase was determined by denaturing polyacrylamide gel electrophoresis. The detailed methodologies are as follows.

5.1.4.1A. Isolation of Bacillus circulans from Turbinaria conoides and Lipase Production

For isolation of epiphytic bacteria, marine macroalga *T. conoides* samples obtained from the Gulf of Mannar were rinsed with sterile seawater, and a small portion of the algal surface was scraped with a sterile swab and spread onto the plates of marine agar plates. After incubation at 37 ^{o}C for 24–48 h, the colonies were streaked onto the marine agar slants and maintained for further microbiological and biochemical identification. Standard sampling procedures were followed, and colonies were recovered from marine agar plates (Chakraborty & Paulraj 2008). The

bacterial cultures obtained from the alga were thereafter grown on modified tributyrin slants (0.3% meat extract, 0.5% peptone, 1% NaCl, 0.3% tributyrin, and 0.01% $CaCl_2$. $2H_2O$) to detect the lipolytic activity. Amongst several epiphytic bacteria isolated from this alga, one Bacillus sp. (*B. circulans*) was used for production of lipase due to its higher lipolytic potential. The bacterial cultures belonging to *B. circulans* were inoculated in a 250 ml erlenmeyer flask containing nutrient broth (100 ml). The content was then incubated at 37 ^oC for 24 h under shaking (150 rpm) to raise the inoculum for the enzyme production. The seed culture (100 ml) (1% v/v) of *B. circulans* was grown in sterilized broth, containing (g l^{-1}); $NaNO_3$ 3; K_2HPO_4 0.1; $MgSO_4.7H_2O$ 0.5; KCl 0.5, $FeSO_4.7H_2O$ 0.05, yeast extract 5.0, and micro algal paste (*Isochrysis galabana*, 5%, v/v) at 37 ^oC in a 1000 ml erlenmeyer flask with shaking (150 rpm) up to 72 h. The culture broth was harvested at different time-intervals (0, 5, 10, 24, 29, 34, 48, 53, 58, and 72 h) to determine the optimum time for maximum lipase production. The culture broth was clarified by centrifugation (10,000g for 20 min at 4 ^oC) to recover the supernatant that was filtered (200 ml). This concentrated liquid, referred to as the crude extra cellular lipase solution, was used for further purification.

5.1.4.1B. Purification of lipase from Bacillus Circulans by Ammonium Sulphated Precipitation and Chromatography

The crude lipase thus obtained above was purified to homogeneity by ammonium sulphate precipitation, sephadex G-100 gel filtration, and amberlite IRA-410 (Cl^{-1} form) anion-exchange chromatography. Solid ammonium sulphate was added to the crude enzyme extract in increments of 5% until 70% saturation (w/v) with gentle stirring for 2 h at 4 ^oC, and allowed to stand for 4 h. The precipitates thus obtained were separated by centrifugation (10,000g for 30 min, 4 ^oC), and the supernatant was fractionated with the slow addition of ammonium sulphate to 70 % saturation. The solution was stirred for 1 h at 4 ^oC, and allowed to stand overnight. The precipitated proteins were collected by centrifugation at 10,000g (30 min, 4 ^oC), and the supernatant was discarded. The pellet was dissolved in a minimum volume of Tris–HCl buffer (50 mM, pH 8.0), and dialysed against the same buffer for 18 h to

remove the residual salts, and assayed for lipase activity (Winkler & Stuckmann 1979). The enzyme concentrate obtained from dialysis was loaded onto a sephadex G-100 gel filtration column (2.5 x 120 cm) at a flow rate of 0.5 ml/min. The column was equilibrated and eluted with Tris–HCl buffer (50 mM, pH 8.0) supplemented with $CaCl_2$ (1.0 mM). The eluant fractions showing lipase activity (obtained at the 22–30th fraction) were pooled together, and concentrated by lyophilization. The lyophilized protein was dissolved in distilled water (5 ml), and dialyzed against Tris-HCl buffer (50 mM, pH 8.0). The concentrated protein was further chromatographed on an amberlite IRA-410 (Cl^{-1} form) column (1.5 x 15 cm) pre-equilibrated with Tris-HCl buffer (50 mM, pH 8.0) and the bound proteins were eluted in Tris-HCl (50 mM, pH 8.0) with a linear gradient of increasing concentration of NaCl (0 - 0.5 M) at a flow rate of 0.5 ml/min. The active fractions (obtained at the 35 - 40th fraction) were pooled, and analyzed for lipase activity. The lipase activity of the enzyme was estimated spectrophotometrically using 4-nitrophenyl palmitate (4-NPP) as reported earlier (Winkler & Stuckmann 1979). One activity unit of lipase (LU) was defined as µmole of 4-NP released from hydrolysis of 4-NPP/ml/min by one ml of enzyme under standard assay conditions. The protein concentration was determined by measuring the absorbance at 590 nm using bovine serum albumin (20–150 µg) as a standard (Bradford 1976). The active enzyme fractions were stored at 4 ^{o}C until used for polyacrylamide gel electrophoresis and further enzyme characterization.

5.1.4.1C. Determination of Molecular Weight of the Lipase by Denaturing Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (0.1% w/v SDS–PAGE) was performed on 12% polyacrylamide gel (with 6% stacking gel), following the established procedure (Laemmli 1970), and the apparent molecular mass of proteins was determined with reference to the low-range molecular mass markers (14.4 - 97.4 kDa). The molecular mass markers used were *β*-phosphorylase (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). After migration, the gels were fixed using 7% (v/v) CH_3COOH/CH_3OH, and

submitted to a cycle of staining/destaining with Coomassie Brilliant Blue R-250 (stainer) and 14% (v/v) CH_3COOH/CH_3OH (destainer), respectively. Molecular mass was determined from the plots of log molecular mass (log M) vs migration (R_f) for the series of protein standards. The active lipase fractions were identified by analytical SDS–PAGE of proteins, using 12% gels stained with Coomassie brilliant blue R-250 as detailed elsewhere (Kim *et al.*, 2005). The gels were rinsed with water and stored in acetic acid (7% v/v) (Lee *et al.*, 1993) to develop a dark yellow color. Gel filtration chromatography with sephadex G-75 was used to validate the molecular weight of lipase. The elution volume of the lipase was compared with those of known protein standards (horse heart cytochrome C, 12.4 kDa; soybean trypsin inhibitor, 20.1 kDa; bovine serum albumin, 66 kDa; *β*-phosphorylase, 97.4 kDa), and the molecular masses of standard proteins were plotted against their respective ratios of elution volume to column void volume (V_e/ V_0) to draw the calibration curve, the column void volume (V_0) being the elution volume of blue dextran (2000 kDa).

5.1.4.2. Lipase-Catalyzed Hydrolysis of the Refined Sardine Oil

The refined oil (RO) was hydrolyzed with lipases purified from *B. circulans*, and the total fatty acid content, at various time-intervals (1-6 h), was analyzed. In brief, a reaction mixture containing triglycerides (E_6; 100 g) in PIPES–NaOH buffer (5 ml, 0.15 M, pH 7.0) was stabilized with Triton X-100 (0.3%, v/v), and $CaCl_2$ (0.5 ml, 100 mM), to which lipase was added (500 LU). The contents were placed in a screw-cap round-bottom flask (250 ml) containing glass beads, following the procedure adopted in an earlier experiment with suitable modification (Tanaka *et al.*, 1994). The round-bottom flask was flushed with N_2 and incubated at 37 ± 1 ^oC under shaking (500 rpm). Samples (0.5 ml) from the reaction mixture were withdrawn periodically (1–6 h) for determining the lipase activity. Methanolic KOH (0.5 N, 25 ml) solution was added to the mixture to neutralize the free fatty acids released during hydrolysis. Distilled water (500 ml) was added to the reaction mixture; the lower aqueous layer was acidified to pH 1.0 with HCl (2 N, 20 ml), and the triacylglycerols were extracted with *n*-hexane (100 ml x 2). The lower aqueous layer

was discarded, and the upper *n*-hexane layer, containing triacylglycerol, was further extracted with distilled water (50 ml x 3) to remove free fatty acids (Chakraborty & Paulraj 2008). The upper *n*-hexane layer was concentrated at 40 ^{o}C *in vacuo* to obtain the lipase hydrolyzed sardine oil (LHSO) which was maintained under N_2 at -20 ^{o}C prior to further use. The hydrolysis products of the reaction were monitored by TLC, using silica gel as adsorbent and $CHCl_3$/Me_2O/gl.AcOH (80:20:0.5, v/v/v) as developing solvent system, and visualized by exposure to iodine vapor.

5.1.4.3. Concentration and Purification of Lipase Hydrolyzed Sardine fatty acids (LHRF)

The lipase hydrolyzed sardine fatty acids (LHRF; *Section 5.1.4.2*) was concentrated using optimum method of urea complexation (*from Section 5.1.3.2*; ie., at 4 ^{o}C for 12h in 4:1 urea/fatty acid ratio) to obtain lipase hydrolyzed concentrated sardine fatty acids (LHCFA). LHCFA after converting to its methyl esters (LHCO) using BF_3/MeOH (*as in section 5.1.3.3*) was further purified by optimum method of argentated-silica column chromatographic fractionation which used sequence of *n*-hexane:Me_2O (*Section 5.1.3.4C*) to obtain 7 fractions. The fraction with maximum purity of EPA, DHA and total PUFA (fraction 6 using *n*-hexane:Me_2O 90:10) was designated as lipase hydrolyzed purified sardine methyl esters (LHPO).

5.1.5. Analysis

The protocols for analyzing the fatty acid composition, free fatty acid (FFA) value, TBARS value, peroxide value (P.V.), para-anisidine value (pA.V.), total oxidation value (TOTOX), UV absorption, GC-MS, LC-MS, FTIR and ^{1}D NMR analysis are as discussed in the previous chapters (*Chapter 4*).

5.1.6. Statistical Analysis

Statistical evaluation was carried out with the Statistical Program for Social Sciences 13.0 (SPSS Inc, Chicago, USA, ver. 13.0). Analyses were carried out in triplicate, and the means of all parameters were examined for significance by analysis of variance (ANOVA). The level of significance for all analyses was $p \leq 0.05$.

5.2. Results and Discussion

5.2.1. Yield after Saponification and Extraction of Free Fatty Acids from Refined Oil

Refined oil (RO, 100 g) on saponification yielded 82.4 g of oil in free fatty acid form (ROF; yield with respect to the crude sardine oil = 60.16 %). The reaction mechanism of saponification and hydrolysis is represented in Fig. 5.2.

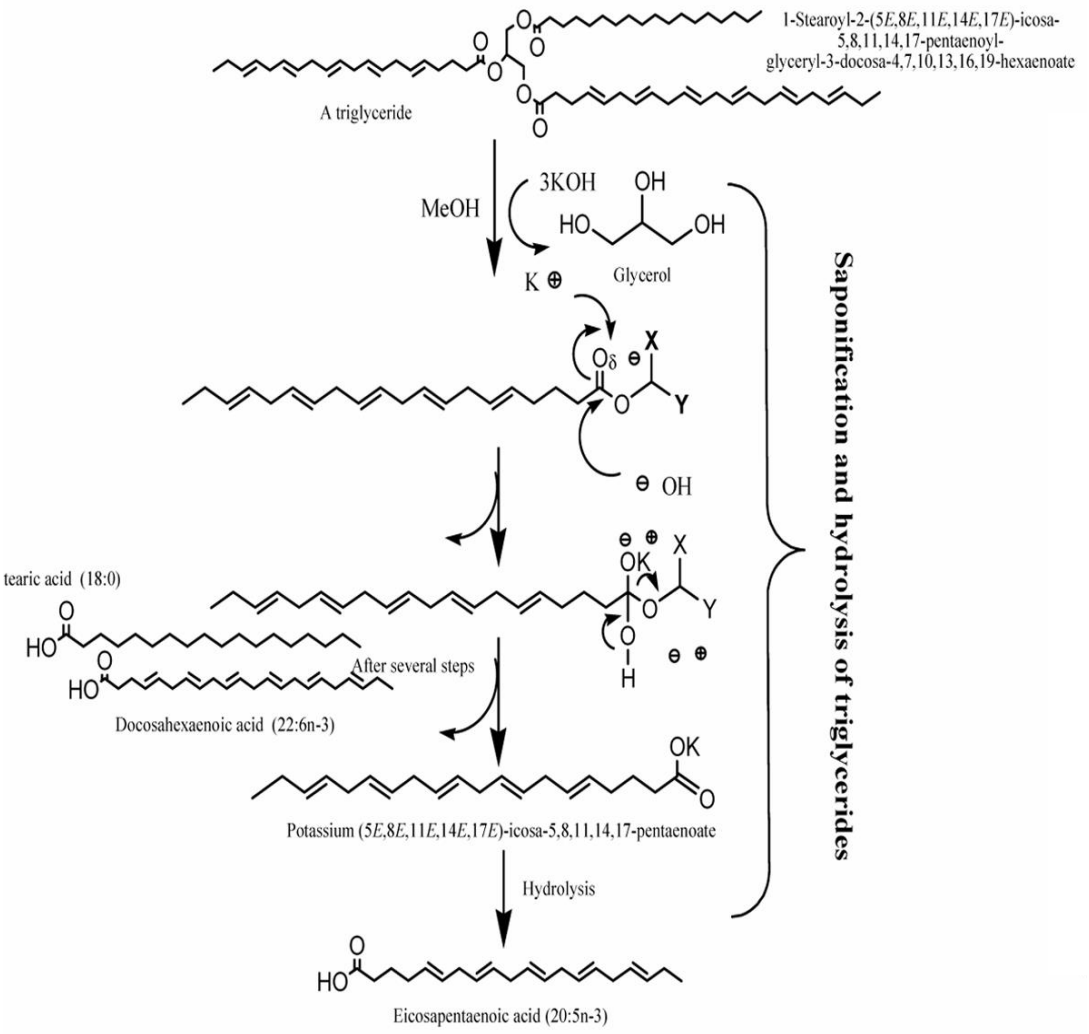


Fig. 5.2. Reaction mechanism showing the saponification and hydrolysis of triglycerides to obtain free fatty acids

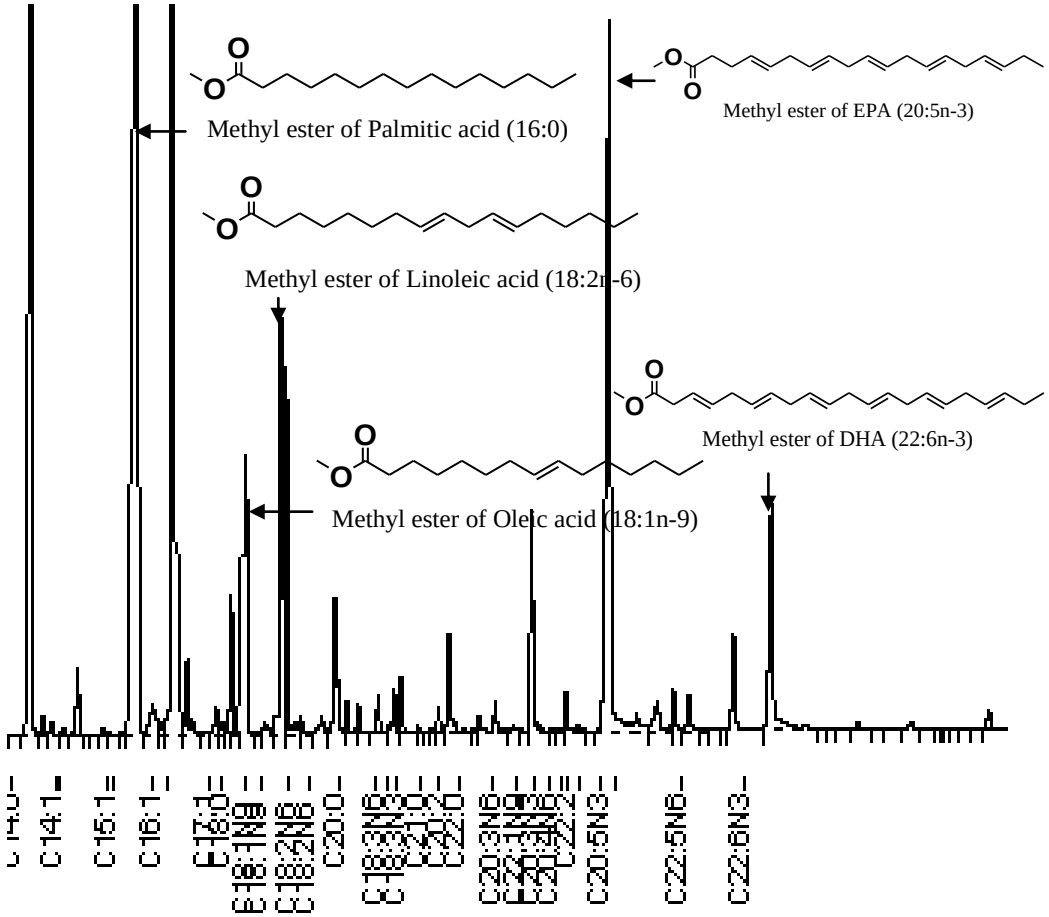


Fig. 5.3. Fatty acid chromatogram of the free fatty acid (FFA) derived from the refined oil (RO)

5.2.2. Preparation of Purified Essential Fatty Acids using Physical and Chemical Techniques

5.2.2.1. Winterization of Refined Sardine Oil (RO)

5.2.2.1A. Recovery, P.V., pA.V., TOTOX, and TBARS Values of the Winterized Oil

The present study indicated that the temperature had influence on the quality characteristics of the sardine oil during winterization. The recovery of the oil was found to be higher when winterized at 4 ^{o}C (> 46 % with respect to the crude oil). Among them, RW_8 (winterization using Me_2O: EtOAc at 7:3 ratio) showed maximum recovery (50.4 % with respect to the crude oil) (Table 5.4).

P.V. was found to be decreased after winterization at -20 ^{o}C ($p>0.05$) with lower values for RW_3 (3 meqO_2/kg) followed by RW_5, RW_4, RW_2 and others. However, the P.V of the oil winterized at 4 ^{o}C did not show any significance as compared to the P.V at -20 ^{o}C ($p<0.05$). A similar trend was followed for pA.V., TOTOX and TBARS values (Table 5.4).

Table 5.4 Recovery, P.V., pA.V., TOTOX and TBARS values of winterized fatty acids (RW_{1-10})

Codes	Recovery	P.V.	pA.V.	TOTOX	TBARS
RO		5.01 ± 0.5^{a}	10.21 ± 0.02^{a}	20.23 ± 2.02^{a}	3.86 ± 0.39^{a}
RW_1	28.65(47.62)	3.59 ± 0.35^{a}	7.02 ± 0.7^{ab}	14.2 ± 1.4^{b}	2.98 ± 0.3^{a}
RW_2	29.25(48.62)	3.45 ± 0.35^{a}	5.14 ± 0.51^{b}	12.04 ± 1.21^{b}	2.69 ± 0.27^{a}
RW_3	28.47(47.32)	2.98 ± 0.3^{a}	5.01 ± 0.5^{b}	10.97 ± 1.1^{b}	2.68 ± 0.27^{a}
RW_4	30.02(49.9)	3.15 ± 0.31^{a}	6.68 ± 0.68^{b}	12.98 ± 1.3^{b}	2.67 ± 0.27^{a}
RW_5	29.97(49.82)	3.02 ± 0.3^{a}	6.01 ± 0.6^{b}	12.05 ± 1.2^{b}	2.99 ± 0.3^{a}
RW_6	47.65(79.21)	4.56 ± 0.46^{a}	7.15 ± 0.73^{ab}	16.27 ± 1.65^{ab}	2.31 ± 0.23^{a}
RW_7	46.25(76.88)	4.84 ± 0.47^{a}	7.01 ± 0.7^{ab}	16.69 ± 1.64^{ab}	3.68 ± 0.38^{a}
RW_8	50.45(83.86)	4.59 ± 0.46^{a}	7.11 ± 0.71^{ab}	16.29 ± 1.63^{ab}	3.65 ± 0.37^{a}
RW_9	47.54(79.02)	4.25 ± 0.42^{a}	7 ± 0.71^{ab}	15.5 ± 1.55^{ab}	3.98 ± 0.4^{a}
RW_{10}	49.58(82.41)	4.35 ± 0.44^{a}	7.01 ± 0.7^{ab}	15.71 ± 1.58^{ab}	3.54 ± 0.34^{a}

Data are expressed as mean ± standard deviation of three replicates. Means with different superscripts (a, b) in the same row indicates a statistical difference ($p<0.05$). RO– Refined sardine oil in its free fatty acid form; RW_{1-10} – Different winterization modes as discussed in Table 5.1. Recovery is represented as % (w/w) with respect to crude sardine oil. Peroxide value (P.V) is represented as $meqO_2$/kg sample. TBARS value is represented as mg MDA/ kg sample. Recovery (% w/w) of the winterized oil with respect to refined oil is given in parentheses.

5.2.2.1B. Fatty Acid Composition of the Winterized Oil

Winterization works on the theory that at low temperatures, fatty acids which have higher melting point crystallize out and PUFA remain in the liquid form. When a saponified fatty acid solution in organic solvents is cooled, SFA and MUFAs crystallize before PUFAs. The common organic solvents, EtOAc and Me_2O were screened to test their effects on the solvent crystallization process.

Table 5.5A and 5.5B showed the fatty acids profiles of the liquid and solid phase, respectively, cooled at different temperatures (-20 & 4 ^{o}C). The subscript 'a' represents the liquid phase and 'b' represents the solid phase.

SFA content was found to be decreased significantly when EtOAc, Me_2O and their three combinations used (-20 ^{o}C) and was in the order, RW_{3a} (17.7 %) < RW_{2a} (30.4 %) < RW_{4a} (31 %) < RW_{1a} (32.8 %) < RW_{5a} (33.5 %). During winterization at 4 ^{o}C of RO, SFA content decreased significantly to 26.4, 28.5 and 31.0 %, when EtOAc, Me_2O: EtOAc (7:3) and Me_2O were respectively used. MUFA concentrations reduced significantly for RW_{7a}, RW_{9a} and RW_{10a} (< 26%; $p<0.05$) while it showed an increment for RW_{1a}, RW_{2a}, RW_{3a}, RW_{5a}, RW_{6a} and RW_{8a} (> 28%). The higher MUFA concentration was observed for Me_2O: EtOAc (7:3) at -20 ^{o}C with percentage increase of 28% compared with initial value. However, MUFA concentrations did not altered significantly for RW_1, RW_2, RW_4 RW_5, RW_7 and RW_{10} ($p>0.05$).

The ROF on winterization at 4 ^{o}C showed that ∑PUFA increased significantly in Me_2O: EtOAc at 7:3 ratio ($RW_{8a;}$ % increase in 62 %) followed by acetone alone (RW_{7a};

% increase - 59.7 %). It should be noted that an extent of 33.3 % of 16:0 and 6.1 % of 18:0, were crystallized and removed (RW_{8b} Table 5.5B), which contributed to the high purity of PUFA in the liquid fraction (36.1 %). After the winterization process at 4 ^{o}C in EtOAc: Me_2O (7:3), an increase in the share of EPA and DHA was obtained, with EPA to 20.3 %, and DHA to 8.9% purity. A high percent increase of EPA was apparent for RW_{8a} (% increase - 58.2 %). Interestingly, DHA content showed significantly high percent increment for RW_{7a} (135.2 %) compared with RW_{8a} (116.3 %). However, the recovery of fatty acid in the crystallized portion was higher for RW_{7b} ($\sum$PUFA - 23.1 %; Table 5.5B). It is further evident from the higher content of SFA in RW_{8b} (50.4 %) that purification of LC-PUFAS is better through the Me_2O: EtOAc solvent system (7:3). The solubility of EPA was found to be higher in more polar solvents and therefore, EPA was concentrated to a higher extent in the Me_2O: EtOAc solvent system at 7:3 ratio (RW_{8a} 20.3 %) as compared to the other solvents/solvent systems. The percent increase of *n*-3 PUFA and *n*-6 PUFA from the initial value was higher for RW_{8a} (30.4 & 5.6%, respectively). This study at different temperatures of winterization depicts that at the higher temperatures (as at 4 ^{o}C) the recovery was observed, although the concentration efficiency showed a downward trend. Thus, it is evident that the long-chain saturated fatty acids, with higher melting points, crystallizes at 4 ^{0}C and separated as liquid form. Since saturated fatty acids are solid in nature due to the compact packing of its straight chain they got crystallized well in Me_2O: EtOAc solvent system (7:3 ratio, 4 ^{0}C). Due to the higher polarity of FFA, a solvent with medium polarity (EtOAc/Me_2O) was required to increase the solubility of unsaturated FFA in the liquid fraction and thus, enhance the selectivity of crystallization. Earlier study showed that the sardine EPA + DHA improved in a two-step winterization procedure (10 and 4ºC) to 34.5 % (Ganga *et al.*, 1998). In the present study, a rise to 29.3 % in EPA + DHA was observed when Me_2O: EtOAc (7:3) is used. The winterization with EtOAc and Me_2O (used individually) showed an enhancement in *n*-3 PUFA (% increment - 19.9 & 16.0%, respectively) and *n*-6 PUFA (% increment - 16.6 & 9.8%, respectively), from their initial value. However, the use of the mixture of these solvents (RW_{8a} Me_2O: EtOAc 7:3) significantly increased the *n*-3 PUFAs and *n*-6 PUFAs to 30.4 & 5.62 %, respectively. Also, the higher yield of RW_{8a} suggested that the PUFA possesses a high solubility in Me_2O: EtOAc (7:3).

The present study indicated that the temperature had influence on the recovery and PUFA content during winterization. Therefore, the winterization at ambient temperature (4 ^{o}C) offered good separation and recovery of *n*-3 PUFAs.

Table 5.5A Fatty acid composition of the sardine fatty acid (WO) non-crystallized fraction after winterization at -20 and 4 °C (RW_{1a-10a})

Fatty acids	RO	RW_{1a}	RW_{2a}	RW_{3a}	RW_{4a}	RW_{5a}	RW_{6a}	RW_{7a}	RW_{8a}	RW_{9a}	RW_{10a}
Saturated											
14:0	12.95±0.3^{a}	11.91±0.19^{a}	11.41±0.15^{a}	9.12±0.9^{ab}	10.8±0.08^{ab}	13.13±0.31^{a}	7.61±0.06^{b}	10.8±0.08^{ab}	11.64±0.16^{a}	7.17±0.02^{ab}	11.33±0.03^{ac}
15:0	0.76±0.08^{a}	0.67±0.07^{a}	0.7±0.07^{a}	0.41±0.04^{a}	0.63±0.06^{a}	0.63±0.06^{a}	0.68±0.07^{a}	0.63±0.06^{a}	0.66±0.07^{a}	0.56±0.06^{a}	0.84±0.08^{a}
16:0	21.98±0.1^{a}	15.19±.03^{b}	14.05±0.41^{b}	5.21±0.02^{c}	15.68±0.57^{b}	15.23±0.52^{b}	14.41±0.44^{b}	15.68±0.57^{b}	13.19±0.32^{b}	25.41±0.54^{a}	20.01±.02^{a}
17:0	0.8±0.08^{a}	0.6±0.06^{a}	0.44±0.04^{a}	0.25±0.03^{a}	0.62±0.06^{a}	0.43±0.04^{a}	0.46±0.05^{a}	0.62±0.06^{a}	0.42±0.04^{a}	0.58±0.06^{a}	1.05±0.1^{a}
18:0	4.13±0.01^{a}	3.56±0.36^{a}	3.2±0.02^{a}	1.95±0.02^{a}	2.53±0.05^{a}	3.21±0.02^{a}	2.89±0.09^{a}	2.53±0.05^{a}	2.25±0.03^{a}	3.5±0.05^{a}	4.54±0.05^{a}
20:0	0.42±0.04^{a}	0.28±0.03^{a}	0.39±0.04^{a}	0.51±0.05^{a}	0.31±0.03^{a}	0.29±0.03^{a}	0.23±0.02^{a}	0.31±0.03^{a}	0.16±0.02^{a}	0.29±0.03^{a}	0.95±0.1^{a}
22:0	0.26±0.03^{a}	0.53±0.05^{a}	0.14±0.01^{a}	0.12±0.01^{a}	0.41±0.04^{a}	0.54±0.05^{a}	0.08±0.01^{a}	0.41±0.04^{a}	0.1±0.01^{a}	0.3±0.03^{a}	0.03±0^{a}
24:0	0.03±0^{a}	0.03±0^{a}	0.08±0.01^{a}	0.11±0.01^{a}	0.03±0^{a}	0.04±0^{a}	0.04±0^{a}	0.03±0^{a}	0.04±0^{a}	0.04±0^{a}	0.05±0.01^{a}
∑SFA	41.34±0.03^{a}	32.77±0.27^{b}	30.41±3.04^{b}	17.68±0.77^{c}	31.01±0.1^{b}	33.5±0.35^{b}	26.4±0.04^{b}	31.01±3.1^{b}	28.46±0.05^{b}	37.85±0.79^{a}	38.8±0.08^{a}
Monounsaturated											
14:1*n*-7	0.16±0.02^{a}	0.06±0.01^{a}	0.09±0.01^{a}	0.11±0.01^{a}	0.06±0.01^{a}	0.07±0.01^{a}	0.08±0.01^{a}	0.06±0.01^{a}	0.1±0.01^{a}	0.14±0.01^{a}	0.09±0.01^{a}
15:1*n*-7	0.08±0.01^{a}	0.02±0^{a}	0.15±0.02^{a}	0.04±0^{a}	0.06±0.01^{a}	0.03±0^{a}	0.02±0^{a}	0.06±0.01^{a}	0.03±0^{a}	0.11±0.01^{a}	0.02±0^{a}
16:1*n*-7 *trans*	0.04±0^{a}	0.02±0^{a}	0.01±0^{a}	0.02±0^{a}	0.01±0^{a}	0.02±0^{a}	0.02±0^{a}	0.01±0^{a}	0.02±0^{a}	0.02±0^{a}	0.02±0^{a}
16:1*n*-7	13.86±0.39^{ab}	14.08±0.41^{a}	13.52±0.35^{ab}	16.03±0.7^{ab}	11.64±0.16^{ab}	13.84±0.38^{ab}	13.17±0.32^{ab}	11.64±0.16^{ab}	14.33±0.43^{a}	10.17±0.02^{b}	11.54±0.14^{ab}
18:1*n*-9 *trans*	0.02±0^{a}	0.02±0^{a}	0.01±0^{a}	0.01±0^{a}	0.01±0^{a}	0.02±0^{a}	0.01±0^{a}	0.01±0^{a}	0.02±0^{a}	0.01±0^{a}	ND
18:1*n*-9	9.69±0.07^{ab}	11.25±0.13^{ab}	9.6±0.06^{ab}	11.52±0.15^{a}	8.34±0.03^{a}	9.48±0.05^{ab}	13.86±0.39^{ab}	8.34±0.03^{a}	10.58±0.06^{ab}	7.92±0.09^{a}	10.94±0.09^{ab}
20:1*n*-9	0.04±0^{a}	0.69±0.07^{a}	0.67±0.07^{a}	0.96±0.1^{a}	0.69±0.07^{a}	0.67±0.07^{a}	0.67±0.07^{a}	0.69±0.07^{a}	0.73±0.07^{a}	0.39±0.04^{a}	0.72±0.07^{a}
22:1*n*-9	2.47±0.05^{a}	3.78±0.08^{a}	3.79±0.37^{a}	4.94±0.08^{a}	4.26±0.43^{a}	3.75±0.38^{a}	3.75±0.38^{a}	4.26±0.03^{a}	3.85±0.09^{a}	3.21±0.02^{a}	2.16±0.02^{a}
24:1*n*-9	0.51±0.05^{a}	0.17±0.02^{a}	0.23±0.02^{a}	0.76±0.08^{a}	0.03±0^{a}	0.22±0.02^{a}	0.31±0.03^{a}	0.03±0^{a}	0.54±0.05^{a}	0.11±0.01^{a}	0.3±0.03^{a}
∑MUFA	26.88±1.69^{ac}	30.09±0.01^{ab}	28.07±0.81^{a}	34.39±0.44^{b}	25.1±0.51^{ac}	28.1±0.81^{a}	31.89±0.19^{ab}	25.1±0.51^{ac}	30.2±0.02^{ab}	22.08±0.21^{c}	25.79±0.58^{ac}
Polyunsaturated											
18:2*n*6 *trans*	0.05±0.01^{a}	0.01±0^{a}	0.02±0^{a}	0.02±0^{a}	0.01±0^{a}	0.01±0^{a}	0.02±0^{a}	0.01±0^{a}	0.01±0^{a}	0.02±0^{a}	ND
18:2*n*-6	1.93±0.19^{a}	2.3±0.22^{a}	2.22±0.22^{a}	2.88±0.29^{a}	2.21±0.22^{a}	2.23±0.21^{a}	2.24±0.22^{a}	2.21±0.22^{a}	2.32±0.23^{a}	1.85±0.19^{a}	2.02±0.2^{a}
18:3*n*-6	0.61±0.06^{a}	0.52±0.05^{a}	0.5±0.05^{a}	0.76±0.08^{a}	0.44±0.04^{a}	0.49±0.05^{a}	0.5±0.05^{a}	0.44±0.04^{a}	0.69±0.07^{a}	0.33±0.03^{a}	0.46±0.05^{a}
18:3*n*-3	0.26±0.03^{a}	0.78±0.08^{a}	0.34±0.03^{a}	0.39±0.04^{a}	0.21±0.02^{a}	0.33±0.03^{a}	0.29±0.03^{a}	0.21±0.02^{a}	0.29±0.03^{a}	0.72±0.07^{a}	0.08±0.01^{a}
20:2*n*-6	1.22±0.11^{a}	1.53±0.15^{a}	1.55±0.06^{a}	2.07±0.21^{a}	1.67±0.17^{a}	1.48±0.15^{a}	1.51±0.15^{a}	1.67±0.17^{a}	1.56±0.16^{a}	1.53±0.15^{a}	2.18±0.22^{a}
20:3*n*-6	0.3±0.03^{a}	0.12±0.01^{a}	0.54±0.05^{a}	0.14±0.01^{a}	0.12±0.01^{a}	0.14±0.01^{a}	0.44±0.04^{a}	0.12±0.01^{a}	0.43±0.04^{a}	0.11±0.01^{a}	0.19±0.02^{a}
20:4*n*-6	0.44±0.04^{a}	0.76±0.08^{a}	0.14±0.01^{a}	0.88±0.09^{a}	0.7±0.07^{a}	0.67±0.07^{a}	0.67±0.07^{a}	0.7±0.07^{a}	0.62±0.06^{a}	0.46±0.05^{a}	0.61±0.06^{a}
20:5*n*-3 EPA	12.85±0.29^{a}	17.71±0.78^{b}	17.24±0.72^{b}	22.42±0.24^{c}	19.7±0.97^{bc}	18.35±0.83^{bc}	17.83±0.77^{b}	19.7±0.98^{bc}	20.34±0.03^{bc}	13.92±0.39^{a}	15.65±0.57^{ab}
22:5*n*-3	0.47±0.05^{a}	0.75±0.08^{a}	0.81±0.08^{a}	0.24±0.02^{a}	0.79±0.08^{a}	0.63±0.06^{a}	0.86±0.09^{a}	0.79±0.08^{a}	0.88±0.09^{a}	1.1±0.11^{a}	0.67±0.07^{a}
22:6*n*-3 DHA	4.12±0.4^{a}	7.27±0.73^{ab}	7.37±0.74^{ab}	9.6±0.96^{b}	9.69±0.07^{b}	6.88±0.69^{ab}	8.81±0.88^{ab}	9.69±0.97^{b}	8.91±0.89^{a}	8.9±0.89^{b}	9.94±0.09^{b}
∑PUFA	22.25±0.23^{a}	31.75±0.18^{b}	30.73±0.06^{b}	39.4±0.93^{c}	35.54±0.54^{c}	31.21±0.11^{b}	33.17±0.32^{b}	35.54±0.55^{c}	36.05±0.61^{c}	28.94±0.89^{b}	31.8±0.18^{b}
EPA+DHA	16.97±0.77^{a}	24.98±0.5^{b}	24.61±0.46^{b}	32.02±0.2^{c}	29.39±0.94^{c}	25.23±0.52^{b}	26.64±0.66^{b}	29.39±0.94^{c}	29.25±0.93^{c}	22.82±0.28^{b}	25.59±0.56^{b}
∑*n*-3PUFA	17.7±0.45^{a}	26.51±0.65^{b}	25.76±0.58^{b}	32.65±0.27^{c}	30.39±0.04^{c}	26.19±0.62^{b}	27.79±0.78^{bc}	30.39±0.04^{c}	30.42±0.04^{a}	24.64±0.46^{b}	26.34±0.63^{b}
∑n-6PUFA	4.5±0.39^{a}	5.23±0.52^{a}	4.95±0.5^{a}	6.73±0.67^{a}	5.14±0.51^{a}	5.01±0.5^{a}	5.36±0.54^{a}	5.14±0.51^{a}	5.62±0.56^{a}	4.28±0.03^{a}	5.46±0.55^{a}
∑*n*-3/∑*n*-6	3.93±0.03^{a}	5.07±0.51^{a}	5.2±0.52^{a}	4.85±0.09^{a}	5.91±0.59^{a}	5.23±0.02^{a}	5.18±0.52^{a}	5.91±0.09^{a}	5.41±0.54^{a}	5.76±0.58^{a}	4.82±0.08^{a}
∑*n*-6/∑*n*-3	0.25±0.94^{a}	0.2±0.02^{a}	0.19±0.02^{a}	0.21±0.02^{a}	0.17±0.02^{a}	0.19±0.02^{a}	0.19±0.02^{a}	0.17±0.02^{a}	0.18±0.02^{a}	0.17±0.02^{a}	0.21±0.02^{a}
∑PUFA/∑SFA	0.54±0.05^{a}	0.97±0.1^{b}	1.01±0.1^{b}	2.23±0.22^{b}	1.15±0.12^{b}	0.93±0.09^{b}	1.26±0.13^{b}	1.15±0.12^{b}	1.27±0.13^{b}	0.76±0.08^{a}	0.82±0.08^{a}
∑TRANS	0.11±0.01^{a}	0.05±0.01^{a}	0.04±0^{a}	0.05±0.01^{a}	0.03±0^{a}	0.05±0.01^{a}	0.05±0.01^{a}	0.03±0^{a}	0.05±0.01^{a}	0.05±0.01^{a}	0.02±0^{a}

e Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA polyunsaturated fatty acids. Means with different superscripts (a, b, c, d) in the same row indicates a statistical difference ($p<0.05$). ND: not detected

Table 5.5B Fatty acid composition of the sardine fatty acid (WO) crystallized fraction after winterization at -20 and 4 °C (RW_{1b-10b})

	RW_{1b}	RW_{2b}	RW_{3b}	RW_{4b}	RW_{5b}	$RW6_b$	RW_{7b}	RW_{8b}	RW_{9b}	RW_{10b}
Saturated										
14:0	12.33±0.23^{a}	12.17±0.21^{a}	12.38±0.24^{a}	13.7±0.37^{a}	11.27±0.13^{ac}	13.59±0.36^{ac}	11.97±0.2^{ac}	8.26±0.83^{a}	15.88±0.59^{c}	11.27±0.13^{ac}
15:0	0.69±0.07^{a}	0.82±0.08^{a}	0.74±0.07^{a}	0.8±0.08^{a}	0.77±0.08^{a}	0.88±0.09^{a}	0.82±0.08^{a}	0.85±0.09^{a}	0.72±0.07^{a}	0.77±0.08^{a}
16:0	25.05±0.51^{a}	25.6±0.54^{a}	20.72±0.07^{a}	22.86±0.29^{a}	23.15±0.33^{a}	22.49±0.25^{a}	29.04±0.9^{b}	33.31±0.33^{b}	28.91±0.88^{b}	23.15±0.33^{a}
17:0	0.81±0.08^{a}	0.86±0.09^{a}	0.79±0.08^{a}	0.55±0.06^{a}	0.86±0.09^{a}	1.01±0.1^{a}	0.91±0.09^{a}	0.84±0.08^{a}	0.64±0.06^{a}	0.86±0.09^{a}
18:0	5.22±0.52^{a}	5.97±0.6^{a}	1.35±0.14^{a}	4.83±0.48^{a}	6.71±0.67^{a}	5.64±0.56^{a}	6.62±0.66^{a}	6.13±0.61^{a}	5.53±0.55^{a}	6.71±0.67^{a}
20:0	0.39±0.04^{a}	0.56±0.06^{a}	0.36±0.04^{a}	0.18±0.02^{a}	0.38±0.04^{a}	0.34±0.03^{a}	0.45±0.05^{a}	0.73±0.07^{a}	0.83±0.08^{a}	0.38±0.04^{a}
22:0	0.15±0.02^{a}	0.32±0.03^{a}	0.26±0.03^{a}	0.09±0.01^{a}	0.1±0.01^{a}	0.13±0.01^{a}	0.06±0.01^{a}	0.21±0.02^{a}	0.04±0^{a}	0.1±0.01^{a}
24:0	0.09±0.01^{a}	0.02±0^{a}	0.03±0^{a}	ND	0.04±0^{a}	0.1±0.01^{a}	0.01±0^{a}	0.03±0^{a}	0.06±0.01^{a}	0.04±0^{a}
∑SFA	44.73±0.47^{a}	46.32±0.63^{a}	36.63±0.66^{b}	43.01±0.3^{a}	43.28±0.33^{a}	44.18±0.42^{a}	49.88±0.99^{c}	50.36±0.04^{c}	52.61±0.26^{c}	43.28±0.33^{a}
Monounsaturated										
14:1*n*-7	0.06±0.01^{a}	0.09±0.01^{a}	0.07±0.01^{a}	0.08±0.01^{a}	0.06±0.01^{a}	0.09±0.01^{a}	0.1±0.01^{a}	0.16±0.02^{a}	0.04±0^{a}	0.06±0.01^{a}
15:1*n*-7	0.1±0.01^{a}	0.07±0.01^{a}	0.11±0.01^{a}	0.11±0.01^{a}	0.05±0.01^{a}	0.19±0.02^{a}	0.03±0^{a}	0.09±0.01^{a}	0.02±0^{a}	0.05±0.01^{a}
16:1*n*7 *trans*	0.02±0^{a}	0.01±0^{a}	0.01±0^{a}	0.03±0^{a}	0.01±0^{a}	0.02±0^{a}	0.03±0^{a}	ND	ND	0.01±0^{a}
16:1*n*-7	11.71±0.17^{a}	11.82±0.18^{a}	13.06±0.31^{a}	14.06±0.41^{a}	12.04±0.1^{a}	10.77±0.08^{ac}	10.22±0.02^{ac}	7.33±0.03^{c}	14.02±0.4^{a}	12.04±0.1^{a}
18:1*n*9 *trans*	0.01±0^{a}	0.02±0^{a}	0.01±0^{a}	0.02±0^{a}	0.02±0^{a}	0.03±0^{a}	0.02±0^{a}	0.02±0^{a}	ND	0.02±0^{a}
18:1*n*-9	8.25±0.83^{a}	8.4±0.84^{a}	9.53±0.95^{a}	13.94±1.39^{a}	11.83±0.18^{ab}	8.06±0.81^{a}	6.12±0.61^{a}	8.18±0.82^{a}	11.39±0.14^{a}	11.83±0.18^{ab}
20:1*n*-9	0.59±0.06^{a}	0.65±0.07^{a}	0.59±0.06^{a}	0.41±0.04^{a}	0.63±0.06^{a}	0.72±0.07^{a}	0.4±0.04^{a}	0.56±0.06^{a}	0.38±0.04^{a}	0.63±0.06^{a}
22:1*n*-9	3.04±0.3^{a}	2.68±0.27^{a}	3.01±0.03^{a}	2.94±0.09^{a}	3.12±0.3^{a}	2.64±0.25^{a}	2.56±0.25^{a}	2.3±0.22^{a}	1.49±0.15^{a}	3.12±0.3^{a}
24:1*n*-9	0.13±0.01^{a}	0.65±0.07^{a}	0.32±0.03^{a}	0.21±0.02^{a}	0.61±0.06^{a}	0.21±0.02^{a}	0.59±0.06^{a}	0.03±0^{a}	0.04±0^{a}	0.61±0.06^{a}
∑MUFA	23.91±0.39^{ac}	24.39±0.44^{ac}	26.71±0.67^{a}	31.8±0.18^{b}	28.37±0.84^{a}	22.73±0.27^{ac}	20.07±0.01^{c}	18.67±0.87^{c}	27.38±0.74^{a}	28.37±0.84^{a}
Polyunsaturated										
18:2*n*6 *trans*	0.02±0^{a}	0.01±0^{a}	0.02±0^{a}	0.01±0^{a}	0.02±0^{a}	0.02±0^{a}	0.02±0^{a}	0.03±0^{a}	0.02±0^{a}	0.02±0^{a}
18:2*n*-6	1.88±0.19^{a}	1.83±0.18^{a}	1.94±0.19^{a}	1.75±0.17^{a}	1.89±0.09^{a}	2.57±0.25^{a}	1.5±0.15^{a}	1.36±0.14^{a}	1.54±0.15^{a}	1.89±0.19^{a}
18:3*n*-6	0.52±0.05^{a}	0.59±0.06^{a}	0.44±0.04^{a}	0.24±0.02^{a}	0.53±0.05^{a}	0.66±0.07^{a}	0.37±0.04^{a}	0.32±0.03^{a}	0.1±0.01^{a}	0.53±0.05^{a}
18:3*n*-3	0.25±0.03^{a}	0.06±0.01^{a}	0.27±0.03^{a}	0.07±0.01^{a}	0.29±0.03^{a}	0.31±0.03^{a}	0.58±0.06^{a}	0.23±0.02^{a}	0.38±0.04^{a}	0.29±0.03^{a}
20:2*n*-6	1.26±0.13^{a}	1.28±0.13^{a}	1.27±0.13^{a}	0.89±0.09^{a}	1.26±0.13^{a}	1.14±0.01^{a}	1.06±0.01^{a}	1.13±0.11^{a}	1.53±0.15^{a}	1.26±0.13^{a}
20:3*n*-6	0.14±0.01^{a}	0.1±0.01^{a}	0.36±0.04^{a}	0.07±0.01^{a}	0.39±0.04^{a}	0.16±0.02^{a}	0.35±0.04^{a}	0.04±0^{a}	0.04±0^{a}	0.39±0.04^{a}
20:4*n*-6	0.53±0.05^{a}	0.45±0.05^{a}	0.49±0.05^{a}	0.17±0.02^{a}	0.55±0.06^{a}	0.47±0.05^{a}	0.53±0.05^{a}	0.37±0.04^{a}	0.12±0.01^{a}	0.55±0.06^{a}
20:5*n*-3 EPA	13.93±0.39^{ab}	13.67±0.37^{ab}	15.67±0.57^{a}	16±0.06^{a}	14.32±0.43^{a}	12.81±0.28^{ab}	12.05±0.21^{ab}	9.88±0.99^{ab}	8.55±0.06^{b}	14.32±0.43^{a}
22:5*n*-3	0.52±0.05^{a}	0.55±0.06^{a}	0.58±0.06^{a}	0.13±0.01^{a}	0.64±0.06^{a}	0.17±0.02^{a}	0.64±0.06^{a}	0.7±0.07^{a}	0.24±0.02^{a}	0.64±0.06^{a}
22:6*n*-3 DHA	4.99±0.5^{a}	4.46±0.05^{a}	5.01±0.5^{a}	3.8±0.38^{a}	6.36±0.64^{a}	4.83±0.48^{a}	5.25±0.53^{a}	5.6±0.56^{b}	5±0.5^{a}	6.36±0.64^{a}
∑PUFA	24.04±0.4^{a}	23±0.3^{ab}	26.05±0.61^{a}	23.13±0.31^{ab}	26.25±0.63^{a}	23.14±0.31^{ab}	22.35±0.24^{ab}	19.66±0.97^{b}	17.52±0.75^{b}	26.25±0.63^{a}
EPA+DHA	18.92±0.89^{ab}	18.13±0.81^{ab}	20.68±0.07^{a}	19.8±0.98^{ab}	20.68±0.07^{a}	17.64±0.76^{ab}	17.3±1.03^{ab}	15.48±0.55^{ab}	13.55±0.36^{b}	20.68±0.07^{a}
∑*n*-3PUFA	19.69±0.97^{a}	18.74±0.87^{ab}	21.53±0.15^{a}	20±2^{a}	21.61±0.16^{a}	18.12±0.81^{ab}	18.52±1085^{a}	16.41±0.06^{a}	14.17±0.42^{b}	21.61±0.16^{a}
∑n-6PUFA	4.33±0.03^{a}	4.25±0.03^{a}	4.5±0.45^{a}	3.12±0.31^{a}	4.62±0.06^{a}	5±0.5^{a}	3.81±0.38^{a}	3.22±0.32^{a}	3.33±0.33^{a}	4.62±0.06^{a}
∑*n*-3/∑*n*-6	4.55±0.46^{a}	4.41±0.44^{a}	4.78±0.48^{a}	6.41±0.04^{a}	4.68±0.47^{a}	3.62±0.06^{a}	4.86±0.49^{a}	5.1±0.51^{a}	4.26±0.43^{a}	4.68±0.07^{a}
∑*n*-6/∑*n*-3	0.22±0.02^{a}	0.23±0.02^{a}	0.21±0.02^{a}	0.16±0.02^{a}	0.21±0.02^{a}	0.28±0.03^{a}	0.21±0.02^{a}	0.2±0.02^{a}	0.24±0.02^{a}	0.21±0.02^{a}
∑PUFA/∑SFA	0.54±0.05^{a}	0.5±0.05^{a}	0.71±0.07^{a}	0.54±0.05^{a}	0.61±0.06^{a}	0.52±0.05^{a}	0.45±0.05^{a}	0.39±0.04^{a}	0.33±0.03^{a}	0.61±0.06^{a}
∑TRANS	0.05±0.01^{a}	0.04±0^{a}	0.04±0^{a}	0.06±0.01^{a}	0.05±0.01^{a}	0.07±0.01^{a}	0.07±0.01^{a}	0.05±0.01^{a}	0.02±0^{a}	0.05±0.01^{a}

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. Means with different superscripts (a, b, c, d) in the same row indicates a statistical difference ($p<0.05$). ND: not detected

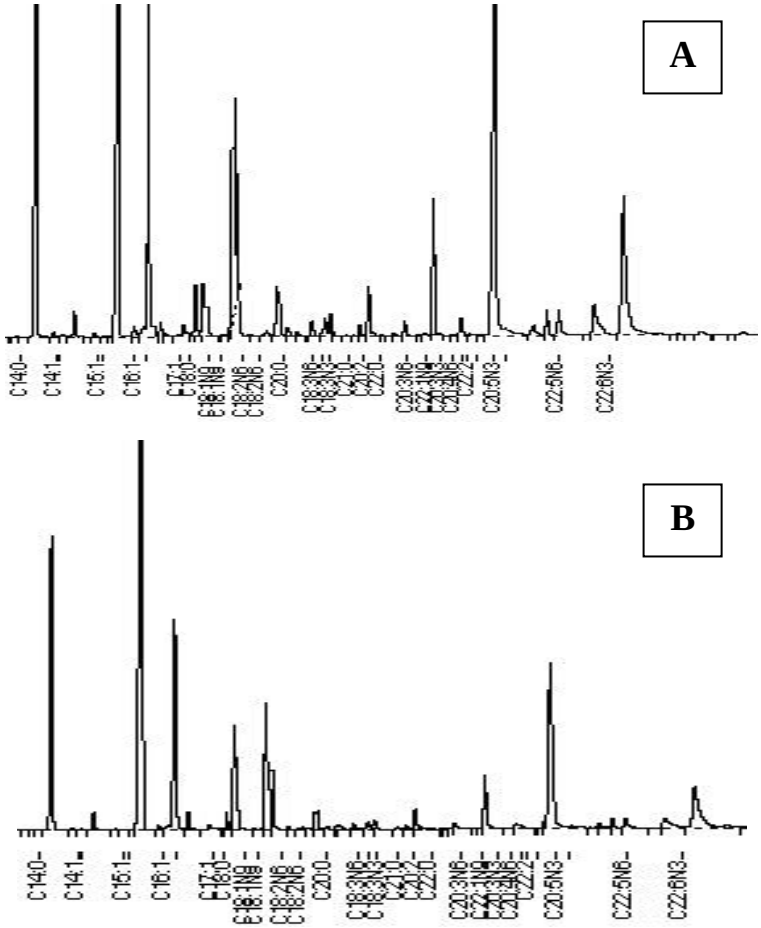


Fig. 5.4. Fatty acid chromatogram of winterized sardine fatty acids WO, (**A**) RW_{8a} and (**B**) RW_{8b}

5.2.2.2. Concentration of Winterized Sardine Fatty Acids Using Amide Complexation

5.2.2.2A. Recovery percent, P.V., pA.V., TOTOX & TBARS values

The recovery of the sardine fatty acids after amide complexation using lower urea:FA ratio showed higher recovery (> 40 % with respect to crude sardine oil E_6). A recovery > 34 % in NUCF was observed when urea: FA at the ratio 4:1 was used, indicating the effective crystallization of the long chain fatty acids in this ratio (Table 5.6).

The P.V. of fatty acid concentrates rose to 10 meqO_2/kg at higher crystallization temperature (10 °C) with 4:1 urea/fatty acid ratio ($p<0.05$) compared to initial value of 4.6 meqO_2/kg. No significant changes in P.V. were apparent during urea fractionation at 4 and -20 °C (Table 5.6). The peroxidation at higher crystallization temperature (10 °C) for urea fractionation was probably due to the release of prooxidants from the oil (Haagsma *et al.*, 1982). P.V. of the U_6 was observed to be below the limit of 10 meqO_2/kg oils for edible oil as indicated in Codex Alimentarius Commission (CAC 2001).

The pA.V. of the fatty acid concentrate was found to be increased during urea complexation and the maximum was observed for U_{13} (pA.V. = 14), followed by U_{12}, U_{13}, U_{15}, U_7, U_9, U_{10}, U_5 and U_4. However, fish oil as indicated by Health Canada (Health Canada, 2009) should have a pA.V. level below 20. The higher pA.V. of > 11 was observed for U_4 and U_7-U_{15}, which showed considerable decomposition of the primary lipid oxidation products (hydroperoxides) into secondary oxidation products (carbonyls).

According to the present results, although the TOTOX value of concentrated fish fatty acids (26.8 for U_6) was in acceptable limits, U_{13} – U_{15} highly exceeded the upper tolerable limit of 26 with higher value for U_{13} (36.1 %).

Higher TBARS values were apparent at the higher temperature of crystallization (U_{13} 3.0 MDAEQ/kg) during the course of urea-fatty acid complexation, thus indicating the formation of secondary lipid oxidation products at a higher temperature.

Table 5.6 Recovery, P.V., pA.V., TOTOX & TBARS of concentrated fatty acids after different modes of urea complexation

Codes	Recovery	P.V.	pA.V.	TOTOX	TBARS
U_0		4.59±0.46^{a}	7.11±0.71^{a}	16.29±0.63^{a}	3.65±0.37^{a}
U_1		4.59±0.46^{a}	7.11±0.71^{a}	16.29±0.63^{a}	3.65±0.37^{a}
U_2	12.5(24.78)	8.02±0.7^{ab}	10.89±0.09^{ab}	26.93±0.69^{b}	2.31±0.22^{a}
U_3	46.8(92.77)	8.01±0.8^{ab}	10.56±0.06^{ab}	26.58±0.66^{b}	2.01±0.2^{a}
U_4	47.12(93.4)	7.98±0.8^{ab}	11.01±0.1^{ab}	26.97±0.7^{b}	2.01±0.2^{a}
U_5	40.26(79.8)	8.21±0.82^{ab}	10.02±0.01^{ab}	26.44±0.64^{b}	2.21±0.22^{a}
U_6	34.8(68.98)	8.4±0.84^{ab}	10±1^{ab}	26.8±.068^{b}	1.63±0.15^{a}
U_7	42.56(84.36)	8.96±0.9^{b}	12.56±0.25^{b}	30.48±0.04^{bc}	1.69±0.17^{a}
U_8	40.01(79.31)	8.44±0.85^{ab}	11.02±0.1^{ab}	27.9±0.79^{b}	1.88±0.18^{a}
U_9	37.45(74.23)	9.98±0.01^{b}	11.56±0.16^{ab}	31.52±0.15^{bc}	1.97±0.2^{a}
U_{10}	30.65(60.75)	7.45±0.75^{ab}	11.05±0.11^{ab}	25.95±0.6^{b}	2.05±0.21^{a}
U_{11}	28.69(56.87)	7.68±0.07^{ab}	10.98±0.1^{ab}	26.34±0.63^{b}	2.15±0.22^{a}
U_{12}	25.69(50.92)	8.01±0.8^{ab}	13.65±0.37^{b}	29.67±0.97^{b}	2.89±0.29^{a}
U_{13}	22.68(44.96)	11.02±0.1^{b}	14.02±0.3^{b}	36.06±0.61^{c}	3.01±0.3^{a}
U_{14}	34.15(67.69)	10±1^{b}	13.21±0.32^{b}	33.21±0.31^{c}	2.01±0.02^{a}
U_{15}	30.25(59.96)	10.41±0.04^{b}	13.52±0.34^{b}	34.34±0.43^{c}	1.98±0.2^{a}

P.V., pA.V., TOTOX, TBARS values as a factor of temperature of crystallization at different urea/fatty acid ratios (2:1, 3:1, and 4:1) *vis-a`-vis* winterized and concentrated sardine fatty acids. Data are expressed as mean ± standard deviation of three replicates. Means with different superscripts (a, b, etc) in the same column indicates a statistical difference ($p<0.05$). U_0 - Winterized sardine fatty acids (RW_{8a}). U_1-U_{15} - Different concentration modes as discussed in Table 5.2. Recovery is represented as % (w/w) with respect to crude sardine oil. Peroxide value (P.V.) is represented as meqO_2/kg sample. TBARS value is represented as mg MDA/ kg sample. Recovery (% w/w) of the CO with respect to WO is given in parentheses.

5.2.2.2B. Fatty Acid Composition of Concentrated Fatty Acids

Urea complexation of fatty acids was used to concentrate PUFAs from SFAs and MUFAs, where urea occludes straight-chain SFAs and MUFAs in the hexagonal moiety, thus eliminating methylene-interrupted PUFAs. This is due to the non-uniformity in these molecules, caused by bends at the location of the olefinic bond (Chakraborty & Paulraj 2007). Urea alone crystallizes in a tightly packed tetragonal structure with channels of 5.67 A^o in diameter. However, in the presence of long straight chain molecules it crystallizes in a hexagonal structure with channels of 8-12 A^o in diameter. The channels formed in the presence of long straight chain molecules are sufficiently large to accommodate aliphatic chains. Different classes of fatty acid composition of non-urea complexed fraction (NUCF) after different modes of urea complexation depicted in Table 5.7A. Similarly, the fatty acid composition of urea complexed fraction (UCF) after different modes of urea complexation is depicted in Table 5.7B.

The winterized sardine fatty acids obtained by optimum method of winterization (RW_{8a}) was subjected to urea crystallization using solvent to obtain

PUFAs of high purity. The effect of urea was evident as no crystals were observed during the crystallization process with EtOH alone at 4 ^{o}C (U_1). No significant difference in fatty acid composition was observed for U_1 (Table 5.7A) ($p<0.05$). It is of note that the crystallization at 4 ^{o}C using EtOH + urea (U_2) resulted in the increase of PUFA at a greater extent to 84.9 % from the initial value of 36 %. A significant increase in both EPA and DHA was also evident for U_{2a}. Even though urea + EtOH increased the purity of PUFAs, the recovery % was not significant (12.5 %) (Table 5.7A). In addition, it is evident that 66.5 % ∑PUFA was lost in the UCF of U_2 (ie., U_{2b}) (Table 5.7B). The crystallization process at 4 ^{o}C using MeOH alone as solvent (U_3) showed 29 % increment in ∑PUFA content with 81.4 % increment in DHA compared to their initial value. Hence the ∑PUFA after MeOH crystallization was recorded to be 46.5 %, mainly contributed by 20:5*n*-3 and 22:6*n*-3. A significant increment in DHA content was observed for U_{3a} (% increase = 81.4 %) ($p<0.05$). A reduction in SFAs (percent reduction - 44.4 %) and MUFAs (percent reduction - 10.5 %) was also observed when using MeOH alone for crystallization. Moreover, it was evident that low amount of ∑PUFA was lost in the UCF U_{3b} (3 %) (Table 5.7B). No significant increment in EPA, DHA and ∑PUFA was observed for urea:WO ratio of 2:1 and 3:1 (U_{4a} & U_{5a}) (Table 5.7A). But a significant increment in the EPA, DHA and ∑PUFA was observed when urea:WO ratio of 4:1 was used (U_{6a}) ($p<0.05$). Hence the effect of urea on the concentration of *n*-3 fatty acids was evident from the present results. It is apparent that the preceding urea process could able to remove nearly all SFAs, most of the MUFAs, and also significantly reduces the linoleic (18:2*n*-6) and α-linolenic (18:3*n*-3) acid levels.

To study the effect of the urea complexation at different temperatures, the complexation at 10 and -20 ^{o}C were also evaluated with three different urea:WO ratios (urea:WO 2:1, 3:1, and 4:1 w/w). Among the three different temperatures of crystallization (4, 10 and -20 ^{o}C) and U/FA ratio (2:1, 3:1 & 4:1) studied, the concentrate obtained from the winterized fatty acids (WO) by urea crystallization at -20 °C using U/WO ratio 4:1 for 12 h (U_{12}) found to contain highest amount of PUFA concentrate (79.9 %), followed by U_6 (4 °C with 4:1 ratio), U_{11} (-20 °C with 3:1 ratio) and U_9 (10 °C with 4:1 ratio) (% PUFA - 73.9, 70.4 & 61.5 %, respectively). In addition, U_6 showed high percent increase in ∑PUFA (2-fold with respect to control ie. WO). Ganga *et al.* (1998) observed the increase in the contents of EPA and DHA (91.3 %) after urea complexation of sardine fatty acids with 17-20 % yield compared to the present study (61.1 % with 34.8 % yield) (Table 5.7A). The purity of 16:1*n*-7, the predominant MUFA, was reduced to 4.8 % at the U/FA ratio of 4:1 and a

temperature of 4 °C. At 10°C, the ∑PUFA was found to be 51.4 – 61.5 %, while at 4 ^{o}C, ∑PUFA was found to be 46.6 – 73.9 %. At -20 ^{o}C, the PUFA content was found to be 48.6 – 79.9 %. However, the recovery of FAs was recorded to be lower (43-51%) at lower (43 – 51 %) at lower temperature (-20 °C) as also at higher temperature (10 °C; 61.4 – 66.0 %). This was apparently due to the poor complexation of SFAs and MUFAs at these temperatures. The EPA purity of the fatty acid concentrate was in the order, U_6 (33.4 %) > U_{12} (33.1 %) > U_{11} (29.8 %) > U_9 (24.6 %) > U_7 (24.1 %) etc whereas the DHA content was in the order, U_{12} (32.6 %) > U_{11} (28 %) > U_6 (27.8 %) > U_9 (24.2 %). Though DHA content was significantly lower for U_6 compared to U_{12} ($p < 0.05$), U_6 showed maximum total recovery of 69 % (with respect to WO Table 5.4). The urea complexation reaction raised the EPA and DHA content of U_6 by 1.6 and 3.1 -fold, respectively (Table 5.7A). Linoleic acid (18:2*n*-6) was found to be concentrated at 4 °C and U/FA ratio of 4:1. It is evident that the degree of saturation in the fatty acid molecule resulted its inclusion in the crystal lattice of urea; the more unsaturated, the less will be the possibility of their inclusion into the urea crystals (Spurvey & Shahidi 2000). The ethyl esters of squid (*Illex argentinus*) visceral oil contained 11.8 % EPA and 14.9 % DHA were purified using urea to 28.2 and 35.6 %, respectively (Hwang & Liang 2001). It is to be noted that less percent of ∑PUFA was lost in the UCF's, when complexation process was carried out at low temperatures (4 and -20^{o}C) as evident from the results in Table 5.7B

In order to study the optimum methodology of urea complexation obtained from the study discussed above different variants of urea/WO (4:1) were studied., (1) RT for 4h, (2) 4 ^{o}C for 24 h and (3) 4 ^{o}C for 36 h (Table 5.7A & 5.7B). The urea complexation at RT for 4h (U_{13}) yielded more ∑PUFA content (84.2 %) followed by U_{15} (4 ^{o}C for 36 h; 70.6 %) and U_{14} (4 ^{o}C for 24h; 62.3 %). But none of these variants studied for urea complexation showed better PUFA composition and recovery compared to U_6 (4 ^{o}C for 12 h) with 73.9 % ∑PUFA. The total SFA content in U_{14} (8.2 %) was significantly higher followed by U_{15} (3.1 %) and U_{13} (1.8 %) ($p<0.05$). U_{13} showed higher EPA and DHA content (35.1 & 36.0 %, respectively), compared to U_{14} and U_{15}. The *n*-3 PUFA obtained after urea complexation is in the order, $U_{13}>U_{15}> U_{14}$. On the basis of these results, the urea complexation at 4 °C using U/WO ratio 4:1 for 12 h (U_6) has been selected as the ideal methodology for the concentration of sardine fatty acids. The fatty acids obtained by this method (U_6) designated as concentrated sardine oil (CO) and was selected for subsequent purification by argentation column chromatography.

Table 5.7A Recovery and percent fatty acid composition of non-urea crystallized fractions (NUCF) obtained by different modes of urea complexation (U_{1a}-U_{6a})

Fatty acids	U_0	U_{1a}	U_{2a}	U_{3a}	U_{4a}	U_{5a}	U_{6a}
Saturated							
14:0	11.64±0.16^a	11.29±0.13^a	0.4±0.04^b	7.38±0.75	0.8±0.08	0.07±0.01	0.97±0.1
15:0	0.66±0.06^a	0.86±0.09^a	0.18±0.02^a	0.42±0.04^a	0.25±0.03^a	0.13±0.01^a	0.16±0.02^a
16:0	13.19±0.32^a	13.21±0.92^a	0.1±0.01^c	5.8±0.58^d	5.65±0.57^d	4.23±0.42^d	0.64±0.06^c
17:0	0.42±0.04^a	0.56±0.11^a	0.44±0.04^a	0.24±0.02^a	0.65±0.07^a	0.51±0.05^a	0.47±0.05^a
18:0	2.25±0.23^a	2.26±0.44^a	0.06±0.01^b	1.74±0.16^a	2.51±0.24^a	1.84±0.19^a	1.94±0.19^a
20:0	0.16±0.02^a	0.17±0.09^a	0.07±0.01^a	0.14±0.01^a	0.14±0.01^a	0.12±0.01^a	0.1±0.01^a
22:0	0.1±0.01^a	0.29±0.03^a	0.06±0.01^a	0.02±0^a	0.14±0.01^a	0.07±0.01^a	0.08±0.01^a
24:0	0.04±0^a	0.04±0.02^a	0.28±0.03^a	0.09±0.01^a	3.25±0.33^b	0.05±0.01^a	0.13±0.01^a
∑SFA	28.46±0.85^a	28.68±0.83^a	1.59±0.16^c	15.83±0.58^d	13.39±0.34^d	7.02±0.07^d	4.49±0.45^{cd}
Monounsaturated							
14:1*n*-7	0.1±0.01^a	0.1±0.01^a	0.13±0.01^a	0.14±0.01^a	8.14±0.81^b	7.8±0.78^b	0.1±0.01^a
15:1*n*-7	0.03±0^a	0.03±0^a	0.01±0^a	0.02±0^a	0.07±0.01^a	0.07±0.01^a	0.06±0.01^a
16:1*n*-7 *trans*	0.02±0^a	0.02±0^a	0.01±0^a	0.05±0.01^a	0.25±0.03^a	0.01±0^a	0.02±0^a
16:1*n*-7	14.33±0.42^a	14.21±0.04^a	1.92±0.19^b	14.48±0.45^a	14.21±0.42^a	15.66±0.57^a	4.8±0.08^b
18:1*n*-9 *trans*	0.02±0^a	0.02±0^a	0.02±0^a	0.02±0^a	0.01±0^a	0.01±0^a	0.01±0^a
18:1*n*-9	10.58±0.05^a	10.68±0.9^a	5.28±0.53^b	7.48±0.75^{ab}	7.54±0.75^{ab}	7.07±0.71^{ab}	5.74±0.57^{bd}
20:1*n*-9	0.73±0.07^a	0.75±0.06^a	0.48±0.05^a	0.93±0.09^a	1.02±0.1^a	1.02±0.1^a	0.04±0^a
22:1*n*-9	3.85±0.39^a	3.82±0.22^a	4.52±0.45^a	3.6±0.36^a	3.25±0.33^a	3.62±0.36^a	4.34±0.43^a
24:1*n*-9	0.54±0.05^a	0.52±0.09^a	0.27±0.03^a	0.3±0.03^a	0.25±0.03^a	0.25±0.03^a	0.38±0.04^a
∑MUFA	30.2±3.02^{ad}	30.15±0.31^a	12.64±0.26^c	27.02±0.7^{ab}	34.74±0.47^d	35.51±0.55^d	15.49±0.55^c
Polyunsaturated							
18:2*n*-6 *trans*	0.01±0^a	0.01±0^a	0.01±0^a	0.03±0^a	0.01±0^a	0.01±0^a	ND
18:2*n*-6	2.32±0.23^a	2.33±0.22^a	3.11±0.31^a	3.22±0.32^a	3.22±0.32^a	3.34±0.33^a	3.09±0.31^a
18:3*n*-6	0.69±0.07^a	0.69±0.04^a	1.28±0.13^a	0.67±0.07^a	0.12±0.01^a	0.47±0.05^a	0.59±0.06^a
18:3*n*-3	0.29±0.03^a	0.29±0.01^a	0.16±0.02^a	0.21±0.02^a	0.05±0.01^a	0.05±0.01^a	0.04±0^a
20:2*n*-6	1.56±0.16^a	1.58±0.22^a	7.1±0.7^b	3.59±0.36^{ab}	3.55±0.36^{ab}	3.66±0.37^{ab}	5.9±0.59^{ab}
20:3*n*-6	0.43±0.04^a	0.43±0.01^a	0.56±0.06^a	0.11±0.01^a	0.13±0.01^a	0.13±0.01^a	0.04±0^a
20:4*n*-6	0.62±0.06^a	0.62±0.02^a	1.32±0.13^a	0.86±0.09^a	0.9±0.09^a	0.9±0.09^a	1.12±0.11^a
20:5*n*-3 EPA	20.34±2.03^a	20.25±0.25^a	34.1±3.41^c	20.38±0.04^a	20.01±2^a	22.68±0.27^a	33.37±0.34^c
22:5*n*-3	0.88±0.09^a	0.88±0.14^a	1.77±0.18^a	1.29±0.13^a	1.44±0.14^a	1.44±0.14^a	2.03±0.2^a
22:6*n*-3 DHA	8.91±0.89^a	8.9±0.9^a	35.5±3.54^b	16.16±0.62^c	17.21±0.72^c	19.15±0.92^c	27.76±0.79^{bd}
∑PUFA	36.05±0.61^a	35.98±0.83^a	84.91±0.49^c	46.52±0.65^d	46.64±0.66^d	51.83±0.18^d	73.94±0.39^c
EPA+DHA	29.25±0.93^a	29.15±0.15^a	69.6±0.96^c	36.54±0.65^d	37.22±0.72^d	41.83±0.18^d	61.13±0.11^c
∑*n*-3PUFA	30.42±0.04^a	30.32±0.31^a	71.53±0.15^c	38.04±0.8^d	38.71±0.87^d	43.32±0.32^d	63.2±6033^{ae}
∑n-6PUFA	5.62±0.06^a	5.65±0.51^a	13.37±0.34^b	8.45±0.85^a	7.92±0.09^a	8.5±0.05^a	10.74±0.07^a
∑*n*-3/∑*n*-6	5.41±0.54^a	5.37±0.45^a	5.35±0.54^a	4.5±0.45^a	4.89±0.49^a	5.1±0.51^a	5.88±0.09^a
∑*n*-6/∑*n*-3	0.18±0.02^a	0.19±0.02^a	0.19±0.02^a	0.22±0.02^a	0.2±0.02^a	0.2±0.02^a	0.17±0.02^a
∑PUFA/∑SFA	1.27±0.13^a	1.25±0.07^a	53.4±0.34^b	2.94±0.29^{ac}	3.48±0.35^{ac}	7.38±0.74^c	16.47±0.65^d
∑TRANS	0.05±0.01^a	0.05±0.01^a	0.04±0^a	0.1±0.01^a	0.27±0.03^a	0.03±0^a	0.03±0^a

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. Means with different superscripts (a, b, etc) in the same row indicates a statistical difference ($p<0.05$). ND: not detected

Table 5.7A *continued.* Recovery and percent fatty acid composition of non-urea crystallized fractions (NUCF) obtained by different modes of urea complexation (U_{7a}-U_{12a})

Fatty acids	U_{7a}	U_{8a}	U_{9a}	U_{10a}	U_{11a}	U_{12a}
Saturated						
14:0	3.85±0.39^{a}	7.37±0.74^{a}	4.83±0.48^{a}	7.31±0.73^{a}	2.4±0.24^{a}	0.52±0.05^{b}
15:0	0.16±0.02^{a}	0.38±0.04^{a}	0.28±0.03^{a}	0.51±0.05^{a}	0.17±0.02^{a}	0.01±0^{a}
16:0	1.78±0.18^{c}	2.13±0.21^{c}	2.4±0.24^{c}	11.16±1.12^{a}	0.36±0.04^{c}	0.16±0.02^{c}
17:0	0.45±0.05^{a}	0.37±0.04^{a}	0.33±0.03^{a}	0.62±0.06^{a}	0.4±0.04^{a}	0.28±0.03^{a}
18:0	2.07±0.21^{a}	1.81±0.18^{a}	0.23±0.02^{a}	2.19±0.22^{a}	0.07±0.01^{b}	1.49±0.15^{a}
20:0	0.58±0.06^{a}	0.68±0.07^{a}	0.12±0.01^{a}	0.47±0.05^{a}	0.11±0.01^{a}	0.1±0.01^{a}
22:0	0.53±0.05^{a}	0.42±0.04^{a}	0.08±0.01^{a}	0.09±0.01^{a}	0.51±0.05^{a}	0.1±0.01^{a}
24:0	0.16±0.02^{a}	0.08±0.01^{a}	0.12±0.01^{a}	0.19±0.02^{a}	0.12±0.01^{a}	0.15±0.02^{a}
∑SFA	9.58±0.06^{d}	13.24±0.32^{d}	8.39±0.84^{d}	22.54±0.25^{a}	4.14±0.41^{cd}	2.81±0.28^{c}
Monounsaturated						
14:1*n*-7	0.08±0.01^{a}	0.14±0.01^{a}	0.14±0.01^{a}	0.1±0.01^{a}	0.15±0.02^{a}	0.13±0.01^{a}
15:1*n*-7	0.05±0.01^{a}	0.06±0.01^{a}	0.03±0^{a}	0.04±0^{a}	0.04±0^{a}	0.06±0.01^{a}
16:1n7 *trans*	0.03±0^{a}	0.01±0^{a}	0.01±0^{a}	0.01±0^{a}	0.01±0^{a}	0.01±0^{a}
16:1*n*-7	16.12±1.61^{a}	15.9±0.59^{a}	12.41±0.24^{a}	8.21±0.82^{c}	12.78±0.28^{a}	3.98±0.4^{b}
18:1n9 *trans*	0.02±0^{a}	0.03±0^{a}	0.02±0^{a}	0.03±0^{a}	0.01±0^{a}	0.04±0^{a}
18:1*n*-9	6.26±0.63^{ab}	11.13±0.11^{a}	6.29±0.63^{ab}	7.88±0.79^{ab}	6.01±0.6^{ab}	4.25±0.43^{b}
20:1*n*-9	1.04±0.1^{a}	0.92±0.09^{a}	0.91±0.09^{a}	0.66±0.07^{a}	1±0.1^{a}	0.86±0.09^{a}
22:1*n*-9	4.45±0.05^{a}	3.65±0.37^{a}	4.21±0.42^{a}	3.19±0.32^{a}	4.4±0.44^{a}	4.95±0.5^{a}
24:1*n*-9	0.15±0.02^{a}	0.25±0.03^{a}	0.28±0.03^{a}	0.64±0.06^{a}	0.17±0.02^{a}	0.46±0.05^{a}
∑MUFA	28.2±0.82^{b}	32.09±0.21^{c}	24.3±0.43^{b}	20.76±0.08^{b}	24.57±0.46^{b}	14.74±0.47^{c}
Polyunsaturated						
18:2n6 *trans*	0.02±0^{a}	ND	0.01±0^{a}	0.01±0^{a}	0.02±0^{a}	0.03±0^{a}
18:2*n*-6	4.03±0.4^{a}	3.51±0.35^{a}	3.62±0.36^{a}	2.57±0.26^{a}	3.98±0.4^{a}	3.92±0.39^{a}
18:3*n*-6	0.37±0.04^{a}	0.35±0.04^{a}	0.32±0.03^{a}	0.36±0.04^{a}	0.32±0.03^{a}	0.35±0.04^{a}
18:3*n*-3	0.3±0.03^{a}	0.03±0^{a}	0.2±0.02^{a}	0.04±0^{a}	0.22±0.02^{a}	0.15±0.02^{a}
20:2*n*-6	3.6±0.36^{ab}	3.65±0.37^{ab}	4.54±0.45^{ab}	3.9±0.39^{ab}	5.08±0.51^{ab}	6.12±0.61^{ab}
20:3*n*-6	0.08±0.01^{a}	0.13±0.01^{a}	0.44±0.04^{a}	0.09±0.01^{a}	0.16±0.02^{a}	0.1±0.01^{a}
20:4*n*-6	1.11±0.11^{a}	0.89±0.09^{a}	1.01±0.1^{a}	0.73±0.07^{a}	1.15±0.12^{a}	1.31±0.13^{a}
20:5*n*-3 EPA	24.06±0.41^{a}	22.93±0.29^{a}	24.6±2.46^{a}	23.09±0.31^{c}	29.79±0.98^{c}	33.1±0.31^{c}
22:5*n*-3	1.47±0.15^{a}	2.29±0.23^{a}	2.57±0.26^{a}	1.32±0.13^{a}	1.69±0.07^{a}	2.23±0.22^{a}
22:6*n*-3 DHA	16.72±0.68^{c}	17.69±0.77^{c}	24.18±0.43^{d}	16.5±0.65^{c}	28±0.8^{bd}	32.6±0.26^{b}
∑PUFA	51.76±0.18^{d}	51.47±0.15^{d}	61.49±0.15^{c}	48.61±0.86^{d}	70.41±0.04^{c}	79.91±0.99^{c}
EPA+DHA	40.78±0.08^{d}	40.62±0.06^{d}	48.78±0.88^{cd}	39.59±0.96^{d}	57.79±0.78^{c}	65.7±0.57^{c}
∑*n*-3PUFA	42.55±0.27^{d}	42.94±0.3^{d}	51.55±0.16^{de}	40.95±0.1^{d}	59.7±0.97^{e}	68.08±0.81^{c}
∑*n*-6PUFA	9.19±0.92^{a}	8.53±0.05^{a}	9.93±0.99^{a}	7.65±0.77^{a}	10.69±0.07^{a}	11.8±0.18^{a}
∑*n*-3/∑*n*-6	4.63±0.46^{a}	5.03±0.5^{a}	5.19±0.52^{a}	5.35±0.04^{a}	5.58±0.56^{a}	5.77±0.58^{a}
∑*n*-6/∑*n*-3	0.22±0.02^{a}	0.2±0.02^{a}	0.19±0.02^{a}	0.19±0.02^{a}	0.18±0.02^{a}	0.17±0.02^{a}
∑PUFA/∑SFA	5.4±0.54^{ac}	3.89±0.39^{c}	7.33±0.73^{c}	2.16±0.22^{a}	17.01±0.7^{d}	28.44±0.84^{e}
∑TRANS	0.07±0.01^{a}	0.04±0^{a}	0.04±0^{a}	0.05±0.01^{a}	0.04±0^{a}	0.08±0.01^{a}

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. Means with different superscripts (a, b,) in the same row indicates a statistical difference ($p<0.05$). ND: not detected

Table 5.7A *continued* Recovery and percent fatty acid composition of non-urea crystallized fractions (NUCF) obtained by different modes of urea complexation n (U_{13n}-U_{15n})

Fatty acids	U_{13n}	U_{14n}	U_{15n}
Saturated			
14:0	0.03±0^{b}	4.25±0.43^{c}	0.16±0.02^{b}
15:0	0.01±0^{a}	0.06±0.01^{a}	0.17±0.02^{a}
16:0	0.09±0.01^{c}	1.4±0.04^{c}	0.24±0.02^{c}
17:0	0.44±0.04^{a}	0.33±0.03^{a}	0.35±0.04^{a}
18:0	0.98±0.1^{a}	1.94±0.19^{a}	2.04±0.2^{a}
20:0	0.12±0.01^{a}	0.09±0.01^{a}	0.05±0.01^{a}
22:0	0.07±0.01^{a}	0.03±0^{a}	0.03±0^{a}
24:0	0.03±0^{a}	0.14±0.01^{a}	0.1±0.01^{a}
∑SFA	1.77±0.18^{c}	8.24±0.02^{d}	3.14±0.31^{c}
Monounsaturated			
14:1*n*-7	0.17±0.02^{a}	0.13±0.01^{a}	0.11±0.01^{a}
15:1*n*-7	0.05±0.01^{a}	0.05±0.01^{a}	0.07±0.01^{a}
16:1n7 *trans*	0.01±0^{a}	0.03±0^{a}	0.03±0^{a}
16:1*n*-7	1.22±0.12^{b}	15±1.5^{a}	7.49±0.75^{c}
18:1n9 *trans*	0.01±0^{a}	0.05±0.01^{a}	0.01±0^{a}
18:1*n*-9	4.67±0.47^{b}	6.19±0.62^{ab}	9.34±0.93^{a}
20:1*n*-9	0.51±0.05^{a}	0.99±0.1^{a}	0.02±0^{b}
22:1*n*-9	4.9±0.49^{a}	4.1±0.41^{a}	4.65±0.47^{a}
24:1*n*-9	0.18±0.02^{a}	0.39±0.04^{a}	0.23±0.02^{a}
∑MUFA	11.72±0.17^{c}	26.93±0.69^{ab}	21.95±0.2^{b}
Polyunsaturated			
18:2n6 *trans*	0.01±0^{a}	0.03±0^{a}	0.02±0^{a}
18:2*n*-6	2.6±0.26^{a}	3.7±0.37^{a}	3.55±0.36^{a}
18:3*n*-6	0.4±0.04^{a}	0.32±0.03^{a}	0.81±0.08^{a}
18:3*n*-3	0.23±0.02^{a}	0.22±0.02^{a}	0.03±0^{a}
20:2*n*-6	5.68±0.57^{ab}	4.27±0.43^{ab}	5.44±0.54^{ab}
20:3*n*-6	0.57±0.06^{a}	0.13±0.01^{a}	0.07±0.01^{a}
20:4*n*-6	1.27±0.13^{a}	1.04±0.1^{a}	0.17±0.02^{a}
20:5*n*-3 EPA	35.05±0.51^{c}	28.5±0.85^{a}	33.36±0.34^{c}
22:5*n*-3	2.42±0.24^{a}	1.68±0.17^{a}	1.56±0.16^{a}
22:6*n*-3 DHA	35.97±0.6^{b}	22.41±0.24^{d}	25.56±0.56^{d}
∑PUFA	84.2±0.42^{c}	62.3±6.23^{c}	70.57±0.06^{c}
EPA+DHA	71.02±0.1^{c}	50.91±0.09^{cd}	58.92±0.89^{c}
∑*n*-3PUFA	73.67±0.37^{c}	52.81±0.28^{de}	60.51±0.05^{a}
∑*n*-6PUFA	10.52±0.05^{a}	9.46±0.95^{a}	10.04±1^{a}
∑*n*-3/∑*n*-6	7±0.7^{a}	5.58±0.56^{a}	6.03±0.6^{a}
∑*n*-6/∑*n*-3	0.14±0.01^{a}	0.18±0.02^{a}	0.17±0.02^{a}
∑PUFA/∑SFA	47.57±0.76^{c}	7.56±0.76^{c}	22.47±0.25^{a}
∑TRANS	0.03±0^{a}	0.11±0.01^{a}	0.06±0.01^{a}

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids,∑PUFA Total polyunsaturated fatty acids. Means with different superscripts (a, b, c, d) in the same row indicates a statistical difference ($p<0.05$).ND: not detected

Table 5.7B Fatty acid composition of the urea crystallized fractions (UCF) obtained by different modes of urea complexation (U_{2b}-U_{6b})

Fatty acids	U_{2b}	U_{3b}	U_{4b}	U_{5b}	U_{6b}
Saturated					
14:0	1.85±0.2^{a}	1.3±1.3^{a}	1.75±0.75^{a}	1.76±0.76^{a}	15.84±0.58^{a}
15:0	0.2±0.02^{a}	0.02±0.02^{a}	0.15±0.15^{a}	0.15±0.15^{a}	1.68±0.17^{a}
16:0	1.69±0.17^{a}	2.05±0.05^{b}	3.52±0.52^{c}	4.29±0.29^{d}	39.05±0.91^{cd}
17:0	0.26±0.03^{a}	0.09±0.09^{a}	0.16±0.16^{a}	0.16±0.16^{a}	2.21±0.02^{a}
18:0	0.1±0.01^{a}	0.41±0.41^{b}	0.6±0.6^{b}	0.89±0.89^{b}	14.46±0.45^{c}
20:0	0.1±0.01^{a}	0.06±0.06^{a}	0.19±0.19^{a}	0.19±0.19^{a}	3.08±0.31^{a}
22:0	0.09±0.01^{a}	0.01±0.01^{a}	ND	ND	0.96±0.1^{a}
24:0	0.07±0.01^{a}	0.01±0.01^{a}	0.02±0.02^{a}	0.02±0.02^{a}	0.58±0.06^{a}
∑SFA	4.36±0.04^{a}	3.93±0.93^{b}	6.4±0.4^{c}	7.44±0.44^{d}	77.86±0.79^{d}
Monounsaturated					
14:1*n*-7	0.16±0.02^{a}	0.09±0.09^{a}	ND	ND	0.02±0^{a}
15:1*n*-7	0.02±0^{a}	0±0	0.01±0.01^{a}	0.01±0.01^{a}	0.07±0.01^{a}
16:1*n*-7 *trans*	0±0	0±0	0±0	0±0	0.02±0^{a}
16:1*n*-7	9.54±0.95^{ab}	1.34±0.34^{ac}	0.62±0.62^{b}	0.75±0.05^{b}	4.64±0.46^{b}
18:1*n*-9 *trans*	0.02±0^{a}	0±0^{a}	0±0	0±0	0±0
18:1*n*-9	5.7±0.56^{ab}	1.14±1.14^{a}	0.21±0.21^{b}	0.23±0.23^{b}	8.41±0.84^{a}
20:1*n*-9	0.32±0.03^{a}	0.03±0.03^{a}	0.01±0.01^{a}	0.01±0.01^{a}	0.69±0.07^{a}
22:1*n*-9	4.48±0.46^{a}	0.22±0.22^{a}	0.1±0.1^{a}	0.1±0.01^{a}	0.38±0.04^{a}
24:1*n*-9	0.43±0.04^{a}	0.02±0.02^{a}	0.04±0.04^{a}	0.04±0.04^{a}	0.02±0^{a}
∑MUFA	20.67±0.07^{a}	2.85±0.85^{a}	1±0.01^{b}	1.15±0.15^{b}	14.25±0.43
Polyunsaturated					
18:2*n*-6 *trans*	0.02±0^{a}	0±0^{a}	0±0	0±0	0±0
18:2*n*-6	3.8±0.38^{a}	0.23±0.23^{a}	0.05±0.05^{a}	0.05±0.05^{a}	0.73±0.07^{a}
18:3*n*-6	1.04±0.01^{a}	0.06±0.06^{a}	0.03±0.03^{a}	0.03±0.03^{a}	0.43±0.04^{a}
18:3*n*-3	0.12±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}	0.04±0^{a}
20:2*n*-6	5.8±0.57^{a}	0.23±0.23^{a}	0.1±0.1^{a}	0.06±0.06^{a}	0.37±0.04^{a}
20:3*n*-6	0.07±0.01^{a}	0.01±0.01^{a}	0.05±0.05^{a}	0.05±0.05^{a}	0.26±0.03^{a}
20:4*n*-6	1.21±0.12^{a}	0.02±0.02^{a}	0.02±0.02^{a}	0.02±0.02^{a}	0.04±0^{a}
20:5*n*-3 EPA	25.73±0.57^{a}	1.55±0.55^{b}	0.57±0.57^{c}	0.36±0.36^{c}	1.19±0.12^{c}
22:5*n*-3	2.82±0.08^{a}	0.02±0.02^{a}	0.04±0.04^{a}	0.04±0.04^{a}	0.05±0.01^{a}
22:6*n*-3 DHA	25.89±0.59^{a}	0.87±0.87^{bd}	0.36±0.36^{bc}	0.25±0.25^{bc}	0.32±0.03^{a}
∑PUFA	66.5±0.65^{a}	3±3^{b}	1.21±0.21^{e}	0.86±0.06^{e}	3.43±0.34^{cd}
EPA+DHA	51.62±0.16^{a}	2.42±0.42^{b}	0.92±0.92^{c}	0.62±0.62^{c}	1.51±0.15^{d}
∑*n*-3PUFA	54.56±0.46^{a}	2.44±0.44^{b}	0.98±0.98^{c}	0.66±0.66^{d}	1.6±0.16^{d}
∑n-6PUFA	11.92±0.19^{a}	0.55±0.55^{b}	0.25±0.25^{b}	0.2±0.02^{b}	1.83±0.18^{b}
∑*n*-3/∑*n*-6	4.58±0.06^{a}	0.45±0.45^{a}	0.39±0.39^{a}	0.33±0.33^{a}	0.87±0.09^{a}
∑*n*-6/∑*n*-3	0.22±0.02^{a}	0.02±0.02^{a}	0.03±0.03^{a}	0.03±0.03^{a}	1.14±0.11^{a}
∑PUFA/∑SFA	15.25±0.53^{a}	0.08±0.08^{b}	0.02±0.02^{b}	0.01±0.01^{b}	0.04±0^{b}
∑TRANS	0.04±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0.02±0^{a}

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. Means with different superscripts (a, b, c, d) in the same row indicates a statistical difference ($p<0.05$). ND: not detected

Table 5.7B *continued* Fatty acid composition of the urea crystallized fractions (UCF) obtained by different modes of urea complexation (U_{7b}-U_{12b})

Fatty acids	U_{7b}	U_{8b}	U_{9b}	U_{10b}	U_{11b}	U_{12b}
Saturated						
14:0	11.61±0.16^{a}	9.19±0.92^{a}	10±1^{a}	7.75±0.78^{a}	6.51±0.65^{a}	17.6±1.76^{a}
15:0	0.56±0.06^{a}	0.76±0.08^{a}	0.85±0.09^{a}	1.2±0.12^{a}	1.14±0.11^{a}	1.86±0.19^{a}
16:0	8.48±0.05^{e}	23.58±0.36^{b}	25.64±0.56^{b}	45.79±0.58^{d}	40.25±0.03^{cd}	38.66±0.87^{c}
17:0	0.25±0.03^{a}	0.81±0.08^{a}	0.81±0.08^{a}	2.58±0.26^{a}	2.58±0.26^{a}	2.15±0.22^{a}
18:0	1.75±0.18^{a}	5.85±0.09^{b}	5.54±0.05^{b}	18.16±0.82^{c}	17.45±0.75^{c}	12.58±0.26^{c}
20:0	0.37±0.04^{a}	1.15±0.12^{a}	1.21±0.12^{a}	4.19±0.42^{a}	4.19±0.42^{a}	2.51±0.25^{a}
22:0	0.26±0.03^{a}	0.32±0.03^{a}	0.32±0.03^{a}	1.38±0.14^{a}	1.08±0.11^{a}	0.81±0.08^{a}
24:0	0.02±0^{a}	0.06±0.01^{a}	0.01±0^{a}	3.03±0.3^{a}	3.03±0.3^{a}	0.02±0^{a}
∑SFA	23.3±0.33^{e}	41.72±0.17^{b}	44.38±0.44^{b}	84.08±0.41^{d}	76.23±0.62^{d}	76.19±0.62^{d}
Monounsaturated						
14:1*n*-7	0.1±0.01^{a}	0.06±0.01^{a}	0.11±0.01^{a}	0.02±0^{a}	0.03±0^{a}	0.15±0.02^{a}
15:1*n*-7	0.02±0^{a}	0.06±0.01^{a}	0.01±0^{a}	0.02±0^{a}	0.02±0^{a}	0.1±0.01^{a}
16:1*n*-7 *trans*	0.02±0^{a}	0.02±0^{a}	0.25±0.03^{a}	0.01±0^{a}	0.01±0^{a}	0.02±0^{a}
16:1*n*-7	18.17±0.82^{c}	6.19±0.62^{b}	7.04±0.07^{b}	2.78±0.28^{b}	2.65±0.27^{b}	5.5±0.55^{b}
18:1*n*-9 *trans*	0±0	0±0	0.01±0^{a}	0.01±0^{a}	0.01±0^{a}	ND
18:1*n*-9	12.42±0.24^{a}	10.21±0.02^{a}	11.21±0.12^{a}	2.36±0.04^{b}	2.56±0.26^{b}	5.93±0.59^{b}
20:1*n*-9	1.34±0.13^{a}	0.55±0.06^{a}	0.54±0.05^{a}	0.18±0.02^{a}	0.18±0.02^{a}	0.21±0.02^{a}
22:1*n*-9	2.79±0.28^{a}	1.94±0.19^{a}	1.89±0.19^{a}	0.61±0.06^{a}	0.61±0.06^{a}	0.44±0.04^{a}
24:1*n*-9	0.21±0.02^{a}	1.2±0.12^{a}	1.01±0.1^{a}	0.75±0.08^{a}	0.74±0.07^{a}	0.25±0.03^{a}
∑MUFA	35.07±0.51^{a}	20.23±0.02^{d}	22.07±0.21^{a}	6.74±0.07^{b}	6.81±0.68^{b}	12.6±0.26^{b}
Polyunsaturated						
18:2*n*-6 *trans*	0.03±0^{a}	0±0	0±0	0.01±0^{a}	0.01±0^{a}	ND
18:2*n*-6	2.78±0.28^{a}	2.23±0.22^{a}	3.01±0.3^{a}	0.52±0.05^{a}	0.55±0.06^{a}	0.68±0.07^{a}
18:3*n*-6	0.48±0.05^{a}	0.46±0.05^{a}	0.54±0.05^{a}	0.1±0.01^{a}	0.1±0.01^{a}	0.21±0.02^{a}
18:3*n*-3	0.16±0.02^{a}	0.03±0^{a}	0.01±0^{a}	0.03±0^{a}	0.03±0^{a}	0.02±0^{a}
20:2*n*-6	2.75±0.28^{a}	2.63±0.26^{a}	2.69±0.27^{a}	0.39±0.04^{a}	0.39±0.04^{a}	0.38±0.04^{a}
20:3*n*-6	0.09±0.01^{a}	0.11±0.01^{a}	0.15±0.02^{a}	0.03±0^{a}	0.03±0^{a}	0.26±0.03^{a}
20:4*n*-6	0.63±0.06^{a}	0.15±0.02^{a}	0.14±0.01^{a}	0.14±0.01^{a}	0.14±0.01^{a}	0.26±0.03^{a}
20:5*n*-3 EPA	15.65±0.57^{b}	13.13±1.31^{b}	15.44±0.54^{b}	1.58±0.16^{c}	2.36±0.24^{c}	0.78±0.08^{c}
22:5*n*-3	0.86±0.09^{a}	0.77±0.08^{a}	0.87±0.09^{a}	0.05±0.01^{a}	0.05±0.01^{a}	0.06±0.01^{a}
22:6*n*-3 DHA	12.23±0.21^{d}	10.36±0.04^{d}	12.35±0.24^{d}	2.03±0.2^{bc}	3.56±0.36^{bc}	0.56±0.06^{c}
∑PUFA	35.66±.57^{b}	29.87±0.99^{b}	35.2±0.52^{b}	4.88±0.49^{cd}	7.22±0.72^{c}	3.21±0.32^{cd}
EPA+DHA	27.88±0.79^{b}	23.49±0.35^{b}	27.79±0.78^{b}	3.61±0.36^{d}	5.92±0.59^{d}	1.34±0.13^{d}
∑*n*-3PUFA	28.9±0.89^{b}	24.29±0.43^{b}	28.67±0.87^{b}	3.69±0.37^{d}	6±0.6^{cd}	1.42±0.14^{d}
∑n-6PUFA	6.73±0.67^{b}	5.58±0.56^{b}	6.53±0.65^{ab}	1.18±0.12^{b}	1.21±0.12^{b}	1.79±0.18^{b}
∑*n*-3/∑*n*-6	4.29±0.43^{a}	4.35±0.44^{a}	4.39±0.44^{a}	3.13±0.31^{a}	4.96±0.5^{a}	0.79±0.08^{a}
∑*n*-6/∑*n*-3	0.23±0.02^{a}	0.23±0.02^{a}	0.23±0.02^{a}	0.32±0.03^{a}	0.2±0.02^{a}	1.26±0.13^{a}
∑PUFA/∑SFA	1.53±0.15^{b}	0.72±0.07^{b}	0.79±0.08^{b}	0.06±0.01^{b}	0.09±0.01^{b}	0.04±0^{b}
∑TRANS	0.05±0.01^{a}	0.02±0^{a}	0.26±0.03^{a}	0.03±0^{a}	0.03±0^{a}	0.02±0^{a}

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. Means with different superscripts (a, b, c, d) in the same row indicates a statistical difference ($p<0.05$). ND: not detected

Table 5.7B *continued* Fatty acid composition of the urea crystallized fractions (UCF) obtained by different modes of urea complexation (U_{13b}-U_{15b})

Fatty acids	U_{13b}	U_{14b}	U_{15b}
Saturated			
14:0	0.23±0.23^{a}	1.33±1.33^{bc}	2.18±2.18^{d}
15:0	0.02±0.02^{a}	0.07±0.07^{a}	0.15±0.15^{a}
16:0	0.1±0.1^{a}	0.67±0.67^{e}	4.06±4.06^{cd}
17:0	0.03±0.03^{a}	0.01±0.01^{a}	0.15±0.15^{a}
18:0	0.21±0.21^{a}	0.13±0.13^{a}	0.75±0.05^{b}
20:0	0.01±0.01^{a}	0.04±0.04^{a}	0.17±0.17^{a}
22:0	0.01±0.01^{a}	0.03±0.03^{a}	0.05±0.05^{a}
24:0	0.01±0.01^{a}	0.02±0.02^{a}	0.02±0.02^{a}
∑SFA	0.62±0.62^{a}	2.3±0.03^{e}	7.52±0.02^{d}
Monounsaturated			
14:1*n*-7	0.02±0.02^{a}	0.01±0.01^{a}	0.01±0.01^{a}
15:1*n*-7	ND	ND	0.01±0.01^{a}
16:1*n*-7 *trans*	ND	ND	ND
16:1*n*-7	0.9±0.9^{b}	1.55±1.55^{c}	0.75±0.05^{b}
18:1*n*-9 *trans*	ND	ND	ND
18:1*n*-9	0.58±0.58^{b}	0.99±0.99^{ab}	0.2±0.2^{b}
20:1*n*-9	0.09±0.09^{a}	0.09±0.09^{a}	0.05±0.05^{a}
22:1*n*-9	0.55±0.55^{a}	0.27±0.27^{a}	0.1±0.01^{a}
24:1*n*-9	0.04±0.04^{a}	0.04±0.04^{a}	0.01±0.01^{a}
∑MUFA	2.18±0.18^{a}	2.95±0.95^{a}	1.12±0.12^{b}
Polyunsaturated			
18:2*n*-6 *trans*	ND	ND	ND
18:2*n*-6	0.44±0.44^{a}	0.33±0.33^{a}	0.04±0.04^{a}
18:3*n*-6	0.04±0.04^{a}	0.05±0.05^{a}	0.05±0.05^{a}
18:3*n*-3	0.03±0.03^{a}	0.02±0.02^{a}	0.06±0.06^{a}
20:2*n*-6	0.61±0.61^{a}	0.31±0.31^{a}	0.08±0.08^{a}
20:3*n*-6	0.01±0.01^{a}	0.01±0.01^{a}	0.01±0.01^{a}
20:4*n*-6	0.13±0.13^{a}	0.07±0.07^{a}	0.02±0.02^{a}
20:5*n*-3 EPA	2.59±0.59^{a}	1.67±0.67^{b}	0.36±0.36^{cd}
22:5*n*-3	0.28±0.28^{a}	0.12±0.12^{a}	0.05±0.05^{a}
22:6*n*-3 DHA	3.03±0.03^{a}	1.31±0.31^{d}	0.24±0.24^{bc}
∑PUFA	7.16±0.16^{a}	3.89±0.89^{b}	0.9±0.9^{c}
EPA+DHA	5.63±0.63^{a}	2.98±0.98^{b}	0.61±0.61^{c}
∑*n*-3PUFA	5.93±0.93^{a}	3.12±0.12^{b}	0.72±0.72^{c}
∑n-6PUFA	1.22±0.22^{a}	0.77±0.77^{a}	0.19±0.19^{b}
∑*n*-3/∑*n*-6	0.49±0.09^{a}	0.4±0.4^{a}	0.38±0.38^{a}
∑*n*-6/∑*n*-3	0.02±0.02^{a}	0.03±0.03^{a}	0.03±0.03^{a}
∑PUFA/∑SFA	1.16±0.16^{a}	0.17±0.17^{b}	0.01±0.01^{b}
∑TRANS	ND	0.01±0.01^{a}	ND

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. Means with different superscripts (a, b, c, d) in the same row indicates a statistical difference ($p<0.05$).ND: not detected

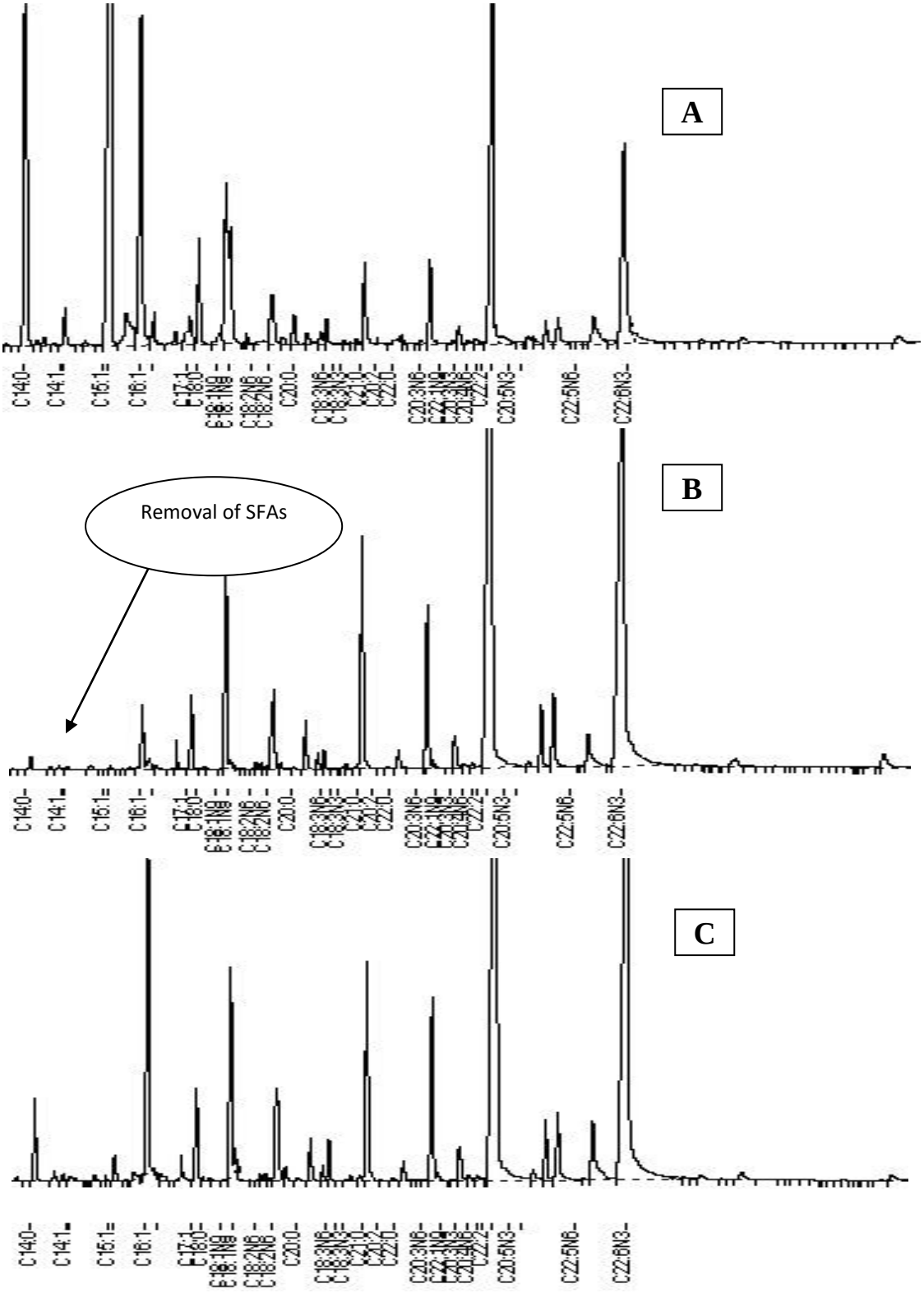


Fig. 5.5.1. Fatty acid chromatogram after urea concentration of the winterized sardine fatty acids, (**A**) U_1; (**B**) U_{2a} and (**C**) U_{2b}

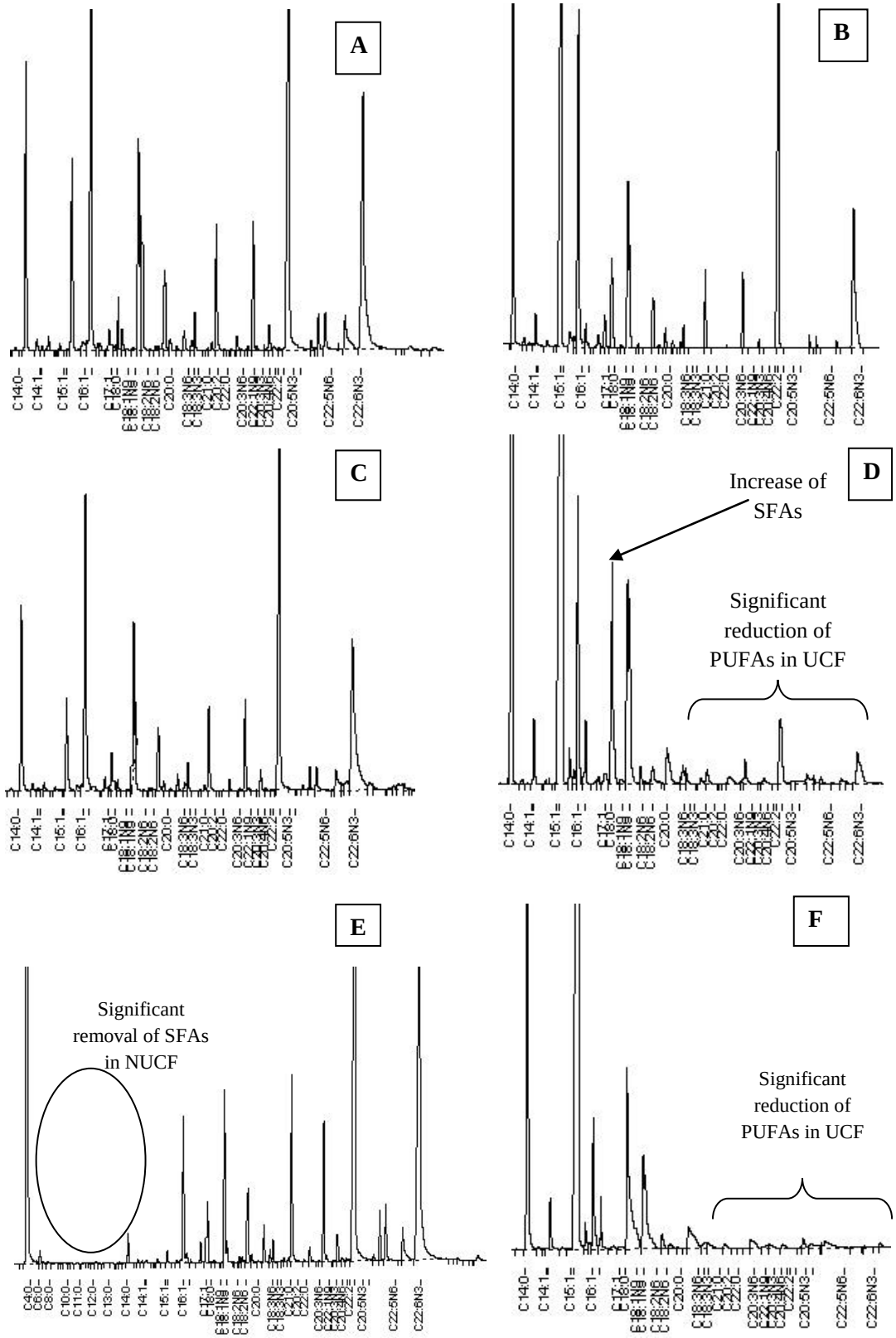


Fig. 5.5.2. Fatty acid chromatogram after urea concentration of the winterized sardine fatty acids., (**A**) U_{3a}; (**B**) U_{3b} and (**C**) U_{5a}; (**D**) U_{5b}; (**E**) U_{6a} and (**F**) U_{6b}

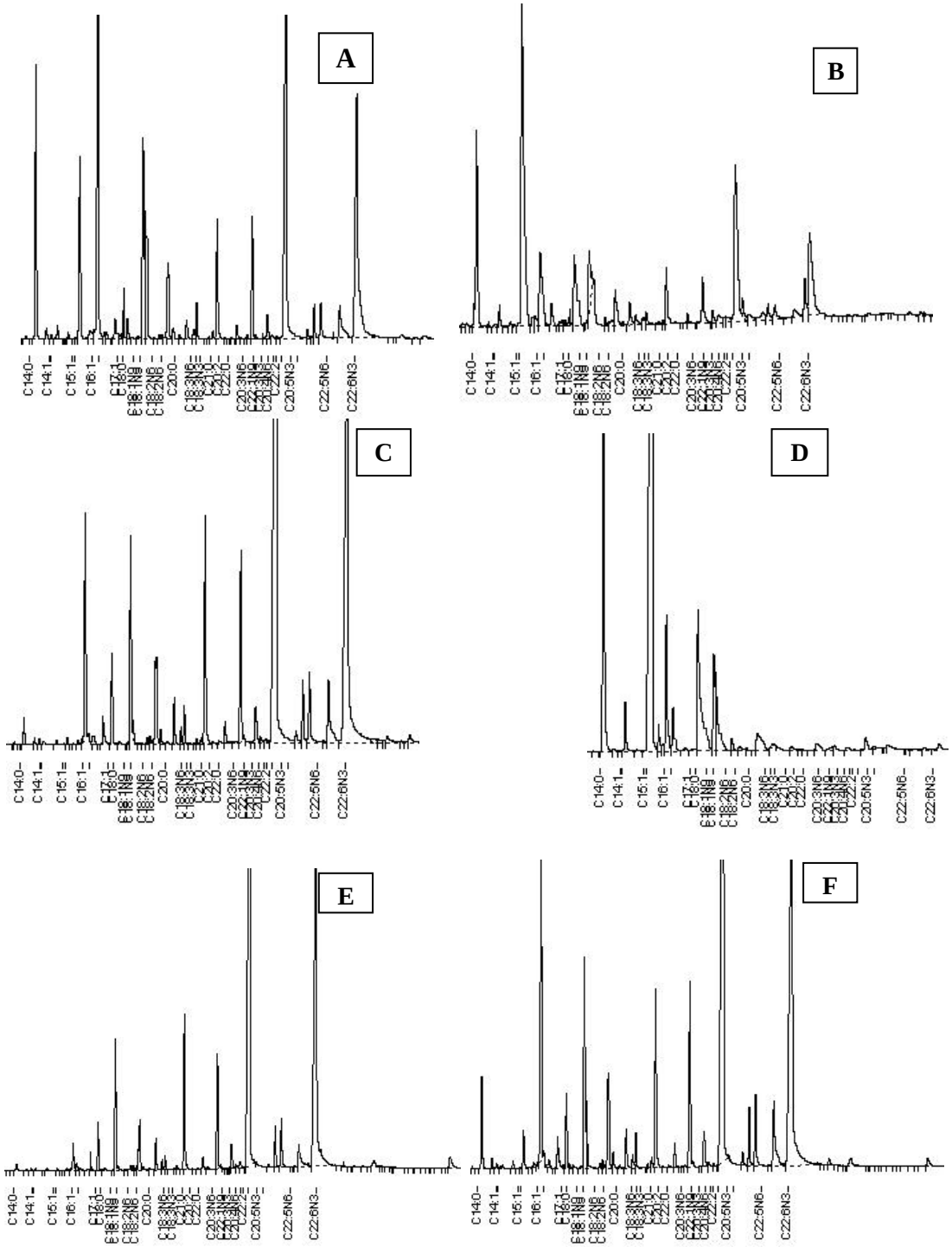


Fig. 5.5.3. Fatty acid chromatogram after urea concentration of the winterized sardine fatty acids., (**A**) U_{8a}; (**B**) U_{8b} and (**C**) U_{12a}; (**D**) U_{12b}; (**E**) U_{13a} and (**F**) U_{13b}

5.2.2.2C. Spectral Analyses of the Winterized and Urea Concentrated Fatty Acids (Rw_{8a} & U_{6a}, respectively) v*is-a-vis* Refined Sardine Oil.

The winterization step was applied when the oil in its free fatty acid form was not clear at room temperature because of the presence of saturated triacylglycerols. It is important to note that these compounds do not negatively affect the oil performance or functionality, but the appearance of the oil is not acceptable to consumers. Thus, the objective of this step is the removal of high temperature melting components such as saturated fatty acids from the mixture of fatty acids. Urea complexation of fatty acids is used to concentrate PUFAs from SFAs and MUFAs, where urea occludes straight-chain compounds such as long-chain saturated and monounsaturated fatty acids in a hexagonal crystalline structure, excluding methylene-interrupted polyunsaturated fatty acids (having the -C=C-C-C=C- moiety) due to the irregularities in their molecules caused by the bends at each double bond. The interfering SFAs and most of the MUFAs (long- and straight-chain molecules) were removed in the form of urea inclusion compound, while the PUFAs remain in solution. Further, as oxidized products do not form urea adducts, the peroxidation of *n*-3 PUFAs could be avoided during the extraction of free acids from fish oil triglycerides. The urea inclusion method allows handling of large quantities of material in a more efficient way than fractional crystallization or selective solvent extraction.

The use of UV spectroscopy in the study of the oils is confined to the systems containing or generating conjugated systems of unsaturation. Conjugated polyenes show a typical absorption at 260-280 nm. Methylene interrupted polyenes such as PUFAs do not show interesting UV absorption until the double bonds migrate to form conjugated systems. It might be possible that during the refining process the double bonds in the PUFAs might change their position and form polyene conjugates (resulting in an increasing in the absorption towards red shift). The winterization process did not demonstrate significant absorption intensities at the area of 200-240 nm (Fig. 5.6A), thereby led us to conclude that there were lesser amount of hydrocarbon functionalities or SFAs with high energy σ- σ transition systems in the

fatty acids. Similarly, no significantly intense absorption peaks between 260-280 nm confirmed that the concentration by urea did not induce the formation of impurities with conjugated olefinic system (Fig. 5.6B), and that there was a reduction in the contents of SFAs with the concomitant increase of methylene interrupted PUFAs.

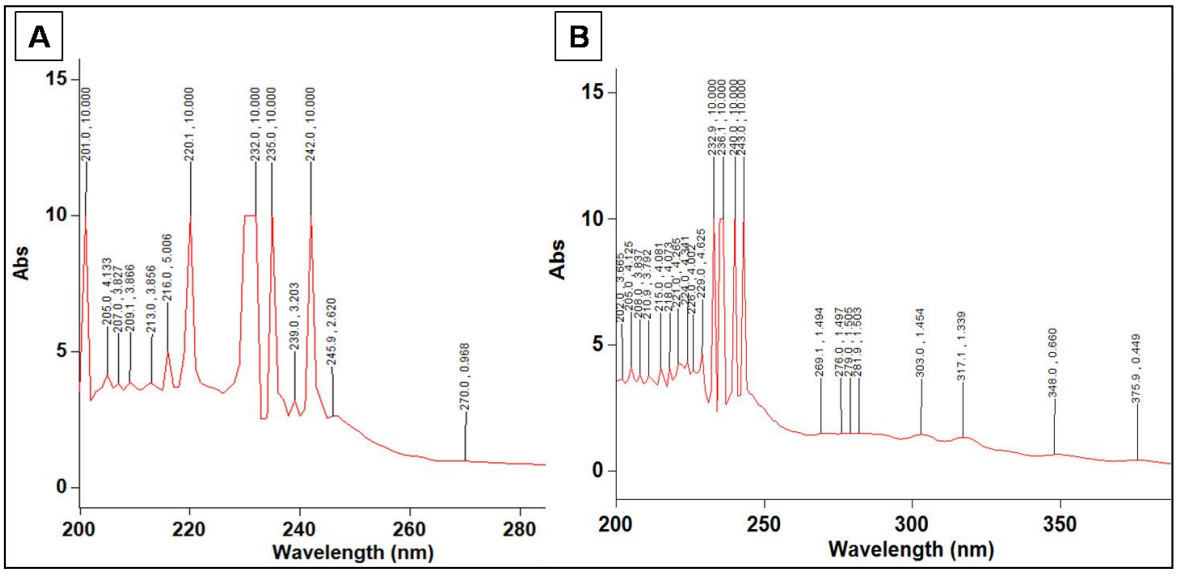


Fig. 5.6. UV-VIS spectra of the (**A**) winterized fatty acids RW_{8a}; (**B**) non-urea complexed fraction (NUCF, U_{6a})

The qualities of the winterized fatty acids (RW_{8a}/WO) and urea concentrated fatty acids (U_{6a}) were studied by ^{1}H-NMR spectroscopy conjugated with ^{13}C-NMR methodology which applies DEPT pulse sequence. The characteristic signals of non-conjugated olefinic protons (-C**H**=C**H**-) in the ^{1}H-NMR spectra appeared at δ 5.2 - 5.6 ppm (m, proton integral, 504.3) (Fig. 5.7 A-C), the multiplet was not baseline resolved from the proton signal at δ5.33 ppm (m), which was assigned to H-2 of the glycerol backbone. A significant increase ($p<0.05$) in the proton integral at this region was apparent after winterization (proton integral, 935.3) (Fig. 5.7 A) and urea concentration (proton integral, 15544). The olefinic proton signals were absent in the urea complexed fraction of fatty acids thereby led us to conclude that the urea fractionation process could effectively eliminate the SFAs in the non-urea complexed fraction (Fig. 5.7 B and 5.7 C). The two double quadruplets at δ 4.95 - 5.1 ppm could be assigned to the protons of the carbon in position 1 (-

C**H**=CH_2) of *n*-1 acyl groups with a proton integral value of 4.5. However, an increase in the proton integral of RW_{8a} (7.2) and U_{6a} (163) showed that these methods could able to significantly concentrate the PUFAs from the refined sardine oil (Fig. 5.7 A & C). The sandwiched CH_2 *bis*-allylic protons (-CH=CH-C**H**$_2$-CH=CH-) were overlapped and displayed at δ 2.74 - 2.92 ppm (multiplets) (proton integral, 303.1). A significant increase in proton integral values was observed for RW_{8a} and U_{6a} (659.6 and 11814.5, respectively) in this region (Fig. 5.7). The peaks at δ 2.36 - 2.42 ppm, which indicate –OCO-C**H**$_2$- *n*-3 fatty acid protons of DHA acyl group (proton integral, 257.4) also increased after winterization and urea concentration (proton integral, 516.5 and 5507, respectively). The peaks at δ 1.92 - 2.15 ppm (proton integral, 275.5) indicated the –C**H**$_2$ group except for DHA acyl group in β position in relation to >C=O group also found to increase after winterization and urea concentration (proton integral, 544.2 and 7469.4, respectively) (Fig. 5.7).

The configuration of the double bond protons was determined by the coupling constant of -CH_2- protons, and the lesser J value signify the presence of *cis* bonds, and the absence of the *trans* double bonds. In general, the coupling constant of –CH_2- protons, which are *trans* in nature, are greater than for *cis* bonds. However the *cis-trans* double bond assignment is difficult to propose because the chemical shift differences of -CH_2- protons of isolated double bonds are small and produce a signal envelope that is very difficult to analyze. The double bond configuration also influences the absorptions of allylic methylene protons. In particular, the -CH_2- protons adjacent to a *cis* double bond as in the present study exhibited a resonance at about δ 2.5 ppm, which is sharper than that produced by a *trans* double bond (less than δ 2 ppm). These results led us to conclude that these steps did not induce the isomeric conversion of *cis* to their *trans* isomers of PUFAs. The fatty acid analyses with authentic standards verified the absence of significant *trans* conformer of the PUFAs after the winterization followed by urea fractionation.

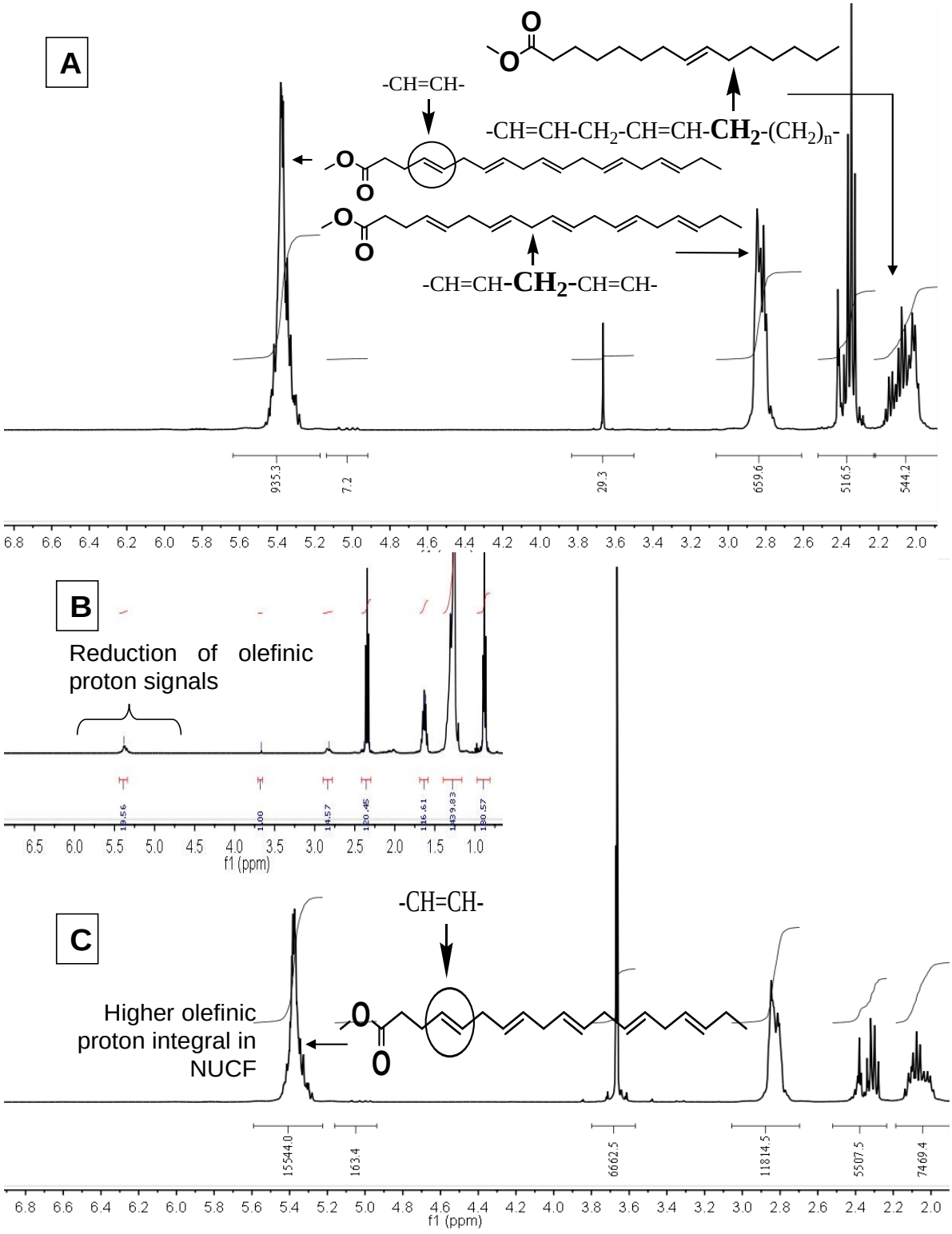


Fig. 5.7. ^{1}H-NMR spectral comparison of (**A**) winterized fatty acid obtained at 4 ^{0}C in Me_2O: EtOAc (7:3, v/v); (**B**) urea complexed fraction of fatty acids with lesser olefinic proton signal; (**C**) non-urea complexed fraction (NUCF) of the fatty acid concentrate.

The resonances of carbons after the process of winterization and urea fractionation illustrated in the ^{13}C-NMR and DEPT spectra were also taken into account to explain the fatty acid resonances. The same sample used for the ^{1}H-NMR experiment was used for the ^{13}C acquisition and Fig 5.8 A-C showed the spectra thus obtained. The characteristic olefinic hydrogen region showed peaks at δ 125-135 ppm as envelope (Fig. 5.8A). Interestingly, the crystallized portion of sardine oil derived by winterization process did not show the presence of intense olefinic system, thereby led us to conclude that this step could able to eliminate the SFAs from the system. These results have been corroborated by the fatty acid analyses of the crystallized portion of the fatty acids derived after winterization *vis-à-vis* the non-crystallized portion of the oil.

The resonances of allylic and *bis*–allylic methylenes, were reported except for the methylene envelope -$(CH_2)_n$, which was indicated at 30 ppm as a whole, and were found to be reduced in the NUCF portion of concentrated sardine oil (Fig. 5.8 C). This led us to infer that the urea fractionation process could able to eliminate SFAs with methylene envelope -$(CH_2)_n$ in their structure. The NUCF portion of concentrated sardine fatty acids (U_{6a}) exhibited the presence of intense olefinic system, thereby led us to conclude that the amide fractionation step could able to concentrate the unsaturated fatty acids. The UCF portion of concentrated sardine fatty acids did not exhibit the presence of intense olefinic system. These results have been corroborated by the fatty acid analyses of the crystallized portion of NUCF portion of concentrated sardine oil *vis-à-vis* the crystallized portion (Table 5.5).

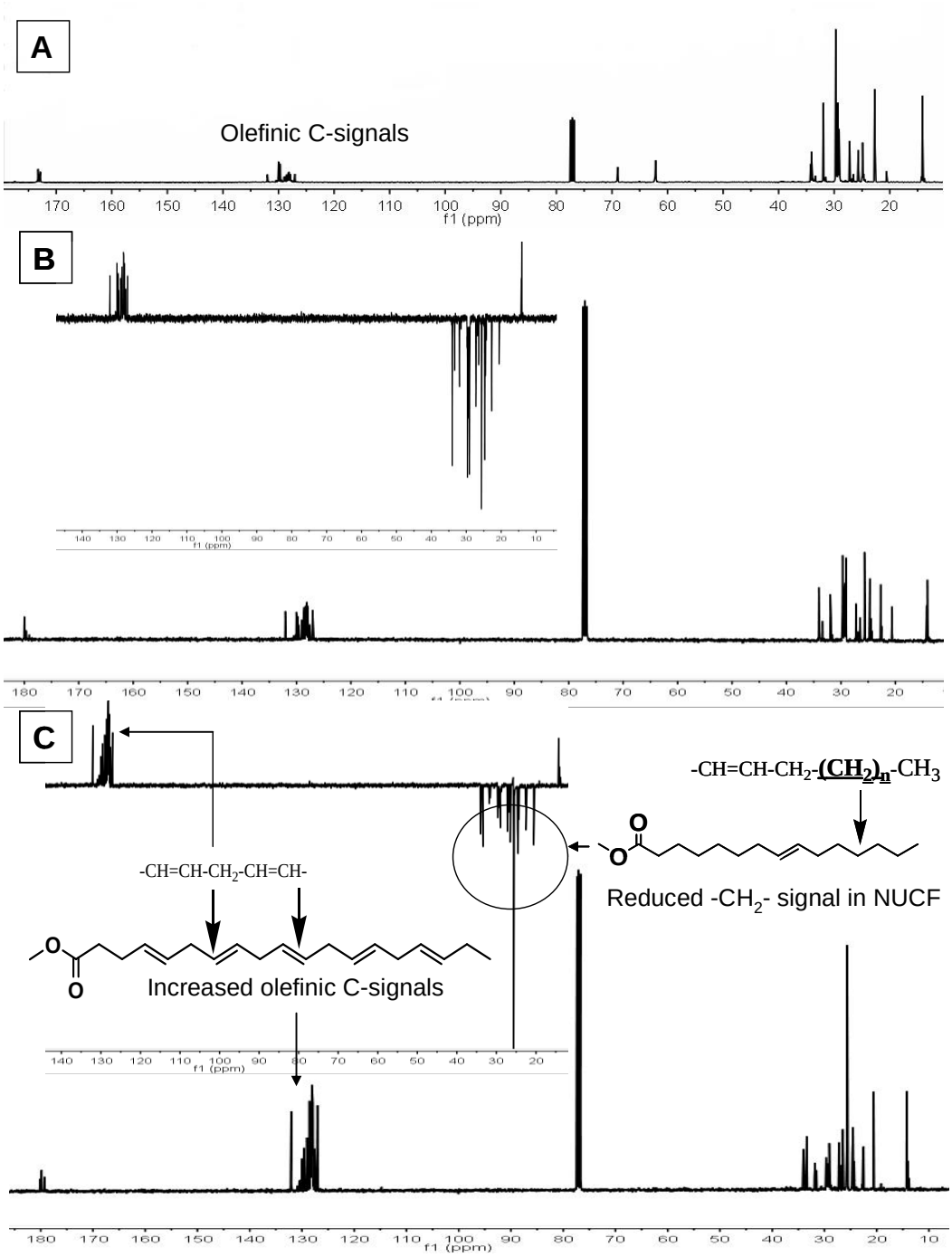


Figure 5.8. ^{13}C-NMR spectra of (**A**) refined sardine oil; (**B**) winterized fatty acid obtained at 4 ^{0}C in Me_2O: EtOAc (7:3, v/v) (inset: $DEPT_{135}$ spectrum); (**C**) fatty acid concentrate obtained after urea complexation at 4 ^{0}C using urea: LCFA ratio of 4:1 (w/w) (inset: $DEPT_{135}$ spectrum showing the increased olefinic C-signals and reduced –CH_2- signals).

The spectral analysis of the oil after the process of winterization was further validated using FT-IR analysis. FT-IR spectra of the NUCF portion of concentrated sardine oil revealed a significant reduction in C-H stretching vibrations (at 2800-3000 cm^{-1}), and bending vibrations at the IR fingerprint region (400-1000 cm^{-1}) as compared to that in control, thereby explaining the absence of hydrocarbon impurities in the sardine oil. An increase in the olefinic double bonds (or the content of unsaturated fatty acids) after urea concentration of the refined sardine oil was also indicated by the presence of characteristic olefinic signals at 1413 and 1283 cm^{-1}.

5.2.2.3. Purification of Concentrated Sardine oil by Argentated Column Chromatography

The purification of fatty acid concentrate (CO; *Section 5.1.3.2*) was achieved by converting it to fatty acid methyl esters (FAMEs), as the esters exhibit more stability and better chromatographic behavior than free fatty acids. The yield of FAMEs after acid catalyzed esterification was 22 % with respect to crude sardine oil E_6 (63.2 % with respect to urea complexed concentrated oil U_{6a}). The formation mechanism of fatty acid methyl esters are shown in the Fig 5.9.

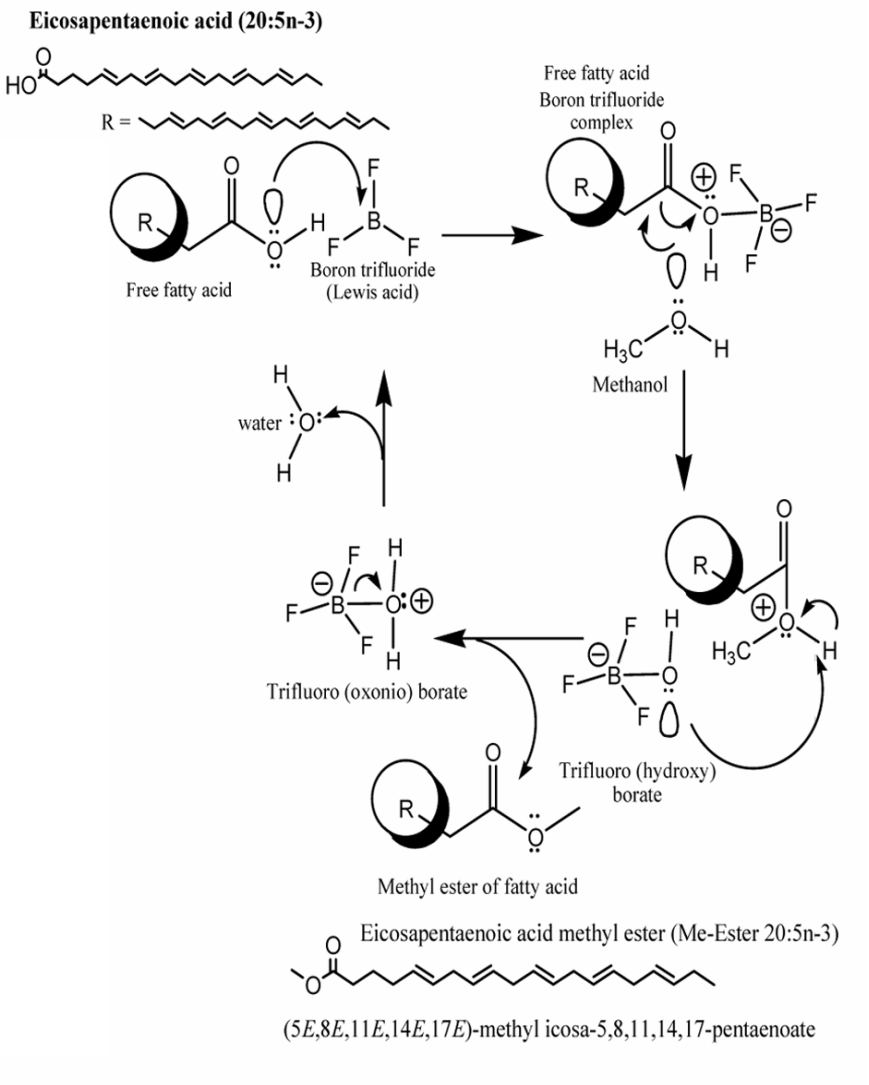


Fig. 5.9. Mechanism showing the formation of fatty acid methyl esters (FAMEs)

Normal pressure column chromatography on neutral alumina/ silica gel impregnated with $AgNO_3$ was evaluated to separate different fatty acids according to the differences in their degree of unsaturation.

5.2.2.3.1. Recovery and Fatty Acid Composition of Different Column Fractions after Argentation Chromatography

5.2.2.3.1A. Argentation Chromatography Using Ag impregnated alumina column

The fatty ester profiles of the various solvent fractions obtained by argentated-alumina chromatography are shown in Table 5.8. The resolving capability of Ag-alumina is mainly attributed to reversible charge transfer complexation of Ag^+ with –

C-C- double bonds of unsaturated compounds (Chakraborty & Paulraj 2007). The fractions FA_4 showed higher yield (31 %) followed by FA_6 (21 %), FA_5 (19.2 %), FA_3 (19 %), FA_7 (4.4 %), FA_2 (1.8 %) and FA_1 (0.4 %). The fraction containing 100% *n*-hexane and 1% Me_2O/*n*-hexane eluted a substantial amount of MUFA esters 16:1*n*-7 (> 14 %), 18:1*n*-9 (> 9.3 %), which associates weakly to the stationary phase. The PUFA content showed 1.3-fold increment in 10% Me_2O/*n*-hexane elution compared to the starting material with 27.5 % recovery. The fraction from 10% Me_2O/*n*-hexane (FA_6) eluted EPA ester of higher purity (45 %) with 27 % recovery. The DHA ester having a larger number of double bonds was found to co-elute with EPA in the second fraction (FA_2) containing 1% acetone/*n*-hexane. FA_6 contain 35.1 % DHA purity compared with 27.8 % of starting material (U_6). It is apparent that the preceding urea complexation removes nearly all SFAs, most of the MUFAs, and also reduces the linoleic (18:2*n*-6) and α-linolenic (18:3*n*-3) acid levels.

The first solvent fraction, i.e., *n*-hexane/ Me_2O (99:1, v/v), was found to elute 16:1*n*-7 (16.1 %) and 18:1*n*-9 (9.4 %) amongst the MUFA methyl esters (Table 5.8). Elution of fatty acids with *n*-hexane/Me_2O (99:2 & 99:3, v/v) substantially eliminated the MUFAs from the urea concentrate. The product contains 20:5*n*-3 methyl esters (43.1 %), *n*-6 fatty acids (82.9 %) after using *n*-hexane/EtOAc (92:8, v/v). FA_6 (*n*-hexane/Me_2O, 90:10, v/v) was eluted with a total of 0.9 % MUFA methyl esters with < 0.3 % of 18:1*n*-9 and 16:1*n*-7 (Table 5.8). However, the predominant fatty ester eluted using this solvent system was found to be 18:3*n*-3 (56.1 %), and 18:4*n*-3 (12.3%) was found to co-elute with it. The fractions eluted with *n*-hexane/ Me_2O (95:5, v/v) were also substantially rich in 18:2*n*-6 (Table 5.8). Solvent fractions with 5% Me_2O/*n*-hexane contained 20:5*n*-3. The fatty acid 20:5*n*-3 was found to be eluted with *n*-hexane/Me_2O (90:10, v/v) with a final purity of 56.1 %. The methyl ester of 22:6*n*-3, with an additional double bond, was found to co-elute (22:6*n*-3, 35.1 %) with 20:5*n*-3 in the fraction containing *n*-hexane/Me_2O (90:10, v/v) as eluting solvent system (Table 5.8). $AgNO_3$– TLC was used to elucidate the progress of purification of PUFAs. The uppermost band (R_f: 0.80–86) of SFAs, and the second band of MUFAs (R_f: 0.46–55) were apparent. FAME up to trienes (18:2*n*-6 and 18:3*n*-3) remained at the base of TLC.

Table 5.8 Recovery (% w/w) and fatty acid composition of the column fractions obtained by argentated alumina column chromatography using *n*-hexane:acetone as eluent

	CO	FA_1	FA_2	FA_3	FA_4	FA_5	FA_6	FA_7
Recovery		0.4	1.8	19	31	19.2	21	4.4
Saturated								
14:0	0.97±0.01^{a}	3.38±0.33^{b}	2.51±0.24^{ab}	1.94±0.19^{ab}	0.47±0.05^{a}	0.03±0^{a}	0.01±0^{a}	0.01±0^{a}
15:0	0.16±0.02^{a}	0.11±0.01^{a}	0.17±0.02^{a}	0.08±0.01^{a}	0.03±0^{a}	0±0	0.01±0^{a}	0.01±0^{a}
16:0	0.64±0.06^{a}	0.73±0.07^{a}	0.04±0^{a}	0.43±0.04^{a}	0.15±0.02^{a}	0.08±0.01^{a}	0.01±0^{a}	0±0^{a}
17:0	0.47±0.05^{a}	0.56±0.06^{a}	0.43±0.04^{a}	0.33±0.03^{a}	0.09±0.01^{a}	0±0	0±0	0±0^{a}
18:0	1.94±0.19^{a}	2.97±0.3^{a}	2.41±0.24^{a}	2.03±0.2^{a}	0.04±0^{b}	0.15±0.02^{ab}	0.01±0^{b}	0±0^{a}
20:0	0.1±0.01^{a}	0.32±0.03^{a}	0.09±0.01^{a}	0.09±0.01^{a}	0.11±0.01^{a}	0.01±0^{a}	0.03±0^{a}	0.02±0^{a}
22:0	0.08±0.01^{a}	0.03±0^{a}	0.08±0.01^{a}	0.08±0.01^{a}	0.03±0^{a}	0.05±0.01^{a}	0.03±0^{a}	0.01±0^{a}
24:0	0.13±0.01^{a}	0.08±0.01^{a}	0.07±0.01^{a}	0.14±0.01^{a}	0.15±0.02^{a}	0.13±0.01^{a}	0.11±0.01^{a}	0.08±0a
∑SFA	4.49±0.05^{a}	8.18±0.82^{a}	5.8±0.58^{a}	5.12±0.51^{a}	1.07±0.11^{ab}	0.45±0.05^{b}	0.21±0.02^{b}	0.13±0a
Monounsaturated								
14:1*n*-7	0.1±0.01^{a}	0.15±0.02^{a}	0.1±0.01^{a}	0.11±0.01^{a}	0.03±0^{a}	0.01±0^{a}	0.04±0^{a}	0.03±0^{a}
15:1*n*-7	0.06±0.01^{a}	0.03±0^{a}	0.56±0.06^{a}	0.12±0.01^{a}	0.05±0.01^{a}	0.03±0^{a}	0.01±0^{a}	0±0a
16:1*n*-7 *trans*	0.02±0^{a}	0.01±0^{a}	0±0	0.02±0^{a}	0.01±0^{a}	0.01±0^{a}	ND	0±0a
16:1*n*-7	4.8±0.48^{ad}	16.13±0.61^{b}	14.52±0.45^{b}	10.72±1.07^{b}	2.93±0.29^{ac}	0.56±0.06^{c}	0.19±0.02^{cd}	0.17±0a
18:1*n*-9 *trans*	0.01±0^{a}	ND	ND	ND	ND	ND	ND	0±0a
18:1*n*-9	5.74±0.58^{a}	9.39±0.93^{a}	5.64±0.55^{a}	5.11±0.51^{a}	2.45±0.24^{ab}	1.31±0.13^{b}	0.33±0.03^{b}	0.28±0a
20:1*n*-9	0.04±0^{a}	0.02±0^{a}	0.04±0^{a}	0.03±0^{a}	0.01±0^{a}	0.01±0^{a}	0.07±0.01^{a}	0.05±0a
22:1*n*-9	4.34±0.43^{a}	5.04±0.5^{a}	4.89±0.49^{a}	4.7±0.47^{a}	0.88±0.09^{b}	0.01±0^{b}	0.01±0^{b}	0±0a
24:1*n*-9	0.38±0.04^{a}	1.14±0.11^{a}	0.29±0.03^{a}	1.9±0.19^{a}	0.78±0.08^{a}	0.61±0.06^{a}	0.24±0.02^{a}	0.23±0a
∑MUFA	15.49±0.05^{a}	31.91±0.19^{b}	26.04±0.6^{b}	22.71±0.27^{b}	7.14±0.71^{c}	2.55±0.26^{d}	0.89±0.09^{d}	0.76±0.01a
Polyunsaturated								
18:2*n*-6 *trans*	ND	0.02±0^{a}	0.01±0^{a}	0.02±0^{a}	0.01±0^{a}	0.01±0^{a}	0.01±0^{a}	0.01±0a
18:2*n*-6	3.09±0.31^{a}	4.38±0.44^{a}	4.1±0.41^{a}	3.96±0.4^{a}	4.06±0.41^{a}	3.44±0.34^{a}	1.98±0.2^{a}	1.08±0.01^{a}
18:3*n*-6	0.59±0.06^{a}	1.17±0.12^{a}	1.06±0.11^{a}	0.97±0.1^{a}	0.42±0.04^{a}	0.24±0.02^{a}	0.08±0.01^{a}	0.05±0^{a}
18:3*n*-3	0.04±0^{a}	0.08±0.01^{a}	0.08±0.01^{a}	0.08±0.01^{a}	0.06±0.01^{a}	0.05±0.01^{a}	0.03±0^{a}	0±0^{a}
20:2*n*-6	5.9±0.59^{a}	4.87±0.49^{a}	5.16±0.52^{a}	5.34±0.53^{a}	5.38±0.54^{a}	4.48±0.45^{a}	2.95±0.3^{a}	2.06±0.02^{a}
20:3*n*-6	0.04±0^{a}	0.6±0.06^{a}	0.11±0.01^{a}	0.11±0.01^{a}	3.08±0.31^{b}	2.46±0.25^{b}	0.03±0^{a}	0.01±0^{a}
20:4*n*-6	1.12±0.11^{a}	1.18±0.12^{a}	1.16±0.12^{a}	0.23±0.02^{a}	0.27±0.03^{a}	0.25±0.03^{a}	0.4±0.04^{a}	0.02±0^{a}
20:5*n*-3 EPA	33.37±0.34^{a}	22.58±0.26^{b}	25.62±0.56^{b}	30.05±0.01^{a}	39.75±0.98^{c}	43.11±0.31^{c}	45.03±0.5^{c}	20.02±0.2^{a}
22:5*n*-3	2.03±0.02^{a}	2.25±0.23^{a}	2.79±0.28^{a}	3.05±0.31^{a}	3.19±0.32^{a}	5.3±0.53^{a}	11.35±0.14^{b}	12.06±0.12^{a}
22:6*n*-3 DHA	27.76±0.78^{a}	14.83±0.48^{b}	22.23±0.22^{a}	24.09±0.41^{a}	29.72±0.97^{a}	34.41±0.44^{b}	35.06±0.5^{b}	48.26±0.48^{a}
∑PUFA	73.94±0.39^{a}	51.96±0.2^{b}	62.32±0.23^{c}	67.9±0.79^{ac}	85.94±0.59^{d}	93.75±0.38^{d}	96.92±0.7^{a}	83.57±0.84^{a}
EPA+DHA	61.13±0.11^{a}	37.41±0.74^{b}	47.85±0.79^{bc}	54.14±0.41^{ac}	69.47±0.95^{a}	77.52±0.75^{d}	80.09±0.08^{d}	68.28±0.68^{a}
∑*n*-3PUFA	63.2±0.33^{a}	39.74±0.98^{b}	50.72±0.08^{bc}	57.27±0.72^{ac}	72.72±0.28^{d}	82.87±0.3^{e}	91.47±0.14f	80.34±0.8^{a}
∑n-6PUFA	10.74±0.07^{ab}	12.2±0.22^{a}	11.59±0.16^{a}	10.61±0.06^{ab}	13.21±0.32^{a}	10.87±0.09^{ab}	5.44±0.54^{b}	3.22±0.03^{a}
∑*n*-3/∑*n*-6	5.88±0.59^{a}	3.26±0.33^{a}	4.38±0.04^{a}	5.4±0.54^{a}	5.5±0.55^{a}	7.62±0.76^{a}	16.81±0.68^{b}	24.95±0.25^{a}
∑*n*-6/∑*n*-3	0.17±0.02^{a}	0.31±0.03^{a}	0.23±0.02^{a}	0.19±0.02^{a}	0.18±0.02^{a}	0.13±0.01^{a}	0.06±0.01^{a}	0.04±0^{a}
∑PUFA/∑SFA	16.47±0.65^{a}	6.35±0.64^{b}	10.74±0.07^{b}	13.26±0.33^{ab}	80.32±0.03^{c}	208.33±0.08^{d}	461.52±0.05^{e}	642.85±0.03^{a}
∑TRANS	0.03±0^{a}	0.03±0^{a}	0.01±0^{a}	0.04±0^{a}	0.02±0^{a}	0.02±0^{a}	0.01±0^{a}	0.01±0^{a}

On the basis of the significance of treatments LSD at the 5% level of significance ($p<0.05$) was computed. Data presented as mean values of three samples (mean ± standard deviation). ND - fatty acids identified on the GC trace but not integrated by the instrument. All other notations are as indicated under Table 5.5.

5.2.2.3.1B. Argentation Chromatography using Ag-silica Column

The fatty ester profiles of the various solvent fractions obtained by the silver-silica gel fractionation of fatty acid esters using *n*-hexane: acetone as eluent is shown in Table 5.9.1. Because the SFAs (C14:0, C16:0) and the MUFAs (C16:1*n*-7, C18:1*n*-9) associate least strongly with the stationary phase, these esters are eluted quantitatively with the FS_1, FS_2 and FS_3 (i.e. the one containing 1, 3 and 5 % Me_2O in *n*-hexane). The column fractions FS_4, FS_5, FS_6 and FS_7 showed significant increase in the ∑PUFA content (> 68 %) compared with initial value of 73.9 %. The fraction from 10% Me_2O/*n*-hexane eluted EPA ester of higher purity (47.6 %) with 31.6 % total recovery. The highly unsaturated EPA (C20:5*n*-3) is barely eluted in the

initial three fractions (1 - 5% Me_2O/*n*-hexane). Though DHA content showed substantially higher purity when eluted with 100% Me_2O (66.7 %) the total recovery was observed to be only 0.6 %. This is certainly because DHA is more hydrophobic relative to EPA and, therefore, has a higher affinity toward the relatively apolar eluent. However, a DHA content of 38.2 % was observed in 10 % Me_2O/*n*-hexane (FS_6) with a high total recovery (31.6 %). As shown in Table 5.9.1, the saturated fatty esters (C14:0, C16:0, C18:0) elute with the first solvent fraction because they do not complex with the Ag^+ ion. Similarly, the monounsaturated esters (C16:1*n*-7, C18:1*n*-9), that complex relatively less strongly with silver, are eluted mostly in the initial solvent fractions (FS_{1-3}). However, as expected, MUFAs eluted fully near the tail end of the 5 % Me_2O fraction and this is confirmed by the significantly low content of C16:1*n*-7 and C18:1*n*-9 in FS_4. The PUFAs started eluting predominantly in FS_5 fraction and its amount was found to be maximum in FS_6 (96.7 %) with 31.6 % of total recovery. After complexation of saturated and less unsaturated FAs by argentation silica column chromatography resulted in EPA and DHA of high purity with high recovery using *n*-hexane:acetone (90:10, % v/v) as eluting solvent (PUFA – 96.7%; EPA – 47.5%; DHA – 38.2%) (Table 5.9.1).

Table 5.9.1 Recovery (% w/w) and fatty acid composition of the column fractions obtained by argentated silica column chromatography using *n*-hexane:acetone as eluent

	CO	FS_1	FS_2	FS_3	FS_4	FS5	FS_6	FS_7
Recovery		6.8	10.2	13	13.8	20.4	31.6	0.6
Saturated								
14:0	0.97±0.05^a	3.41±0.05^b	3.02±0.04^b	3.26±0.03^b	1.68±0^b	0.05±0^c	0.01±0^c	0.06±0^c
15:0	0.16±0.01^a	0.25±0.01^a	0.15±0.01^a	0.14±0^a	0.03±0^b	0.01±0^b	ND	ND
16:0	0.64±0.03^a	0.74±0.03^a	0.89±0.03^a	0.16±0^a	0.07±0^b	ND	ND	ND
17:0	0.47±0.02^a	0.47±0.02^a	0.41±0.02^a	0.35±0.01^a	0.17±0.01^a	0.02±0^b	0.01±0^b	ND
18:0	1.94±0.1^a	5.01±0.05^b	6.19±0.04^b	6.05±0.04^b	1.36±0.02^a	0.18±0.01^a	0.01±0^c	0.06±0^c
20:0	0.1±0.01^a	0.04±0^a	0.04±0^a	0.03±0^a	0.03±0^a	0.03±0^a	0.04±0^a	0.11±0.01^a
22:0	0.08±0^a	0.09±0^a	0.14±0^a	0.07±0^a	0.06±0^a	0.06±0^a	0.04±0^a	0.08±0^a
24:0	0.13±0.01^a	0.16±0.01^a	0.26±0.01^a	0.35±0.01^a	0.18±0.01^a	0.18±0.01^a	0.19±0.01^a	0.22±0.01^a
∑SFA	4.49±0.22^a	10.17±0.18^b	11.1±0.15^b	10.41±0.1^b	3.58±0.05^a	0.53±0.03^c	0.3±0.02^c	0.53±0.03^c
Monounsaturated								
14:1*n*-7	0.1±0.01^a	0.1±0.01^a	0.08±0^a	0.07±0^a	0.04±0^a	0.01±0^a	0.01±0^a	0.06±0^a
15:1*n*-7	0.06±0^a	0.06±0^a	0.06±0^a	0.05±0^a	0.04±0^a	0.03±0^a	0.01±0^a	0.06±0^a
16:1*n*-7 *trans*	0.02±0^a	0.01±0^a	ND	ND	ND	ND	ND	ND
16:1*n*-7	4.8±0.24^a	14.05±0.2^{bc}	15.26±0.18^b	17.14±0.1^b	10.35±0.06^c	0.46±0.02^b	0.43±0.02^b	0.16±0.01^b
18:1*n*-9 *trans*	0.01±0^a	0.01±0^a	0.01±0^a	ND	ND	ND	ND	ND
18:1*n*-9	5.74±0.29^a	9.02±0.16^b	7.89±0.14^b	6.91±0.14^{ab}	1.96±0.1^c	1.79±0.09^c	0.58±0.03^c	0.14±0.01^c
20:1*n*-9	0.04±0^a	0.03±0^a	0.03±0^a	0.25±0^a	0.01±0^a	ND	0.03±0^a	0.08±0^a
22:1*n*-9	4.34±0.22^a	8.56±0.2^b	6.98±0.13^{ab}	5.33±0.1^{ab}	1.01±0.05^b	0.25±0.01^c	0.34±0.02^c	0.68±0.03^c
24:1*n*-9	0.38±0.02^a	0.36±0.02^a	0.37±0.02^a	0.37±0.02^a	0.35±0.02^a	0.34±0.02^a	0.43±0.02^a	0.57±0.03^a
∑MUFA	15.49±0.77^a	32.2±0.59^b	30.68±0.48^b	30.12±0.37^b	13.76±0.23^a	2.88±0.14^c	1.83±0.09^c	1.75±0.09^c
Polyunsaturated								
18:2*n*-6 *trans*	ND	ND	ND	ND	ND	ND	ND	ND
18:2*n*-6	3.09±0.15^a	4.98±0.16^a	3.14±0.16^a	4.01±0.2^a	4.08±0.2^a	4.09±0.2^a	5.28±0.26^a	1.96±0.1^a
18:3*n*-6	0.59±0.03^a	1.21±0.03^a	0.46±0.02^a	0.42±0.02^a	0.28±0.01^a	0.27±0.01^a	0.09±0^a	0.28±0.01^a
18:3*n*-3	0.04±0^a	0.08±0^a	0.03±0^a	0.03±0^a	0.03±0^a	0.03±0^a	0.03±0^a	0.11±0.01^a
20:2*n*-6	5.9±0.3^a	5.02±0.3^a	6.11±0.31^a	6.36±0.32^a	6.42±0.32^a	6.45±0.32^a	3.06±0.15^a	0.97±0.05^b
20:3*n*-6	0.04±0^a	0.18±0.01^a	0.96±0.05^{ab}	1.36±0.07^{ab}	2.01±0.1^b	2.86±0.14^b	0.1±0.01^a	0.22±0.01^{ab}
20:4*n*-6	1.12±0.06^a	1.02±0.05^a	0.86±0.04^a	0.87±0.04^a	0.88±0.04^a	0.88±0.04^a	0.37±0.02^a	0.34±0.02^a
20:5*n*-3 EPA	33.37±1.67^a	19.65±1.7^b	20.01±1.71^b	22.32±1.79^b	31.02±1.81^a	38.85±1.94^e	47.55±2.38^d	24.54±1.23^b
22:5*n*-3	2.03±0.1^a	2.01±0.1^a	2.12±0.11^a	2.86±0.14^a	3.46±0.17^a	4.54±0.23^a	2.05±0.1^a	1.38±0.07^a
22:6*n*-3 DHA	27.76±1.39^a	10.51±1.41^b	10.26±1.41^b	11.68±1.47^b	20.22±1.65^c	37.44±1.87^d	38.15±1.91^d	66.67±0.33^e
∑PUFA	73.94±0.7	44.66±3.75	43.95±0.81	49.91±0.05	68.4±0.32	95.41±0.77	96.68±0.83	96.47±0.82
EPA+DHA	61.13±0.06^a	30.16±0.11^b	30.27±0.13^b	34±0.26^b	51.24±3.46^c	76.29±3.81^d	85.7±4.29^d	91.21±0.56^e
∑*n*-3PUFA	63.2±3.16^a	32.25±3.21^b	32.42±3.23^b	36.89±3.4^b	54.73±3.64^c	80.86±4.04^d	87.78±4.39^{de}	92.7±0.64^e
∑n-6PUFA	10.74±0.54^a	12.41±0.54^a	11.53±0.58^a	13.02±0.65^a	13.67±0.68^a	14.55±0.73^a	8.9±0.45^a	3.77±0.19^b
∑*n*-3/∑*n*-6	5.88±0.29^{ab}	2.6±0.3^a	2.81±0.28^a	2.83±0.26^a	4±0.27^a	5.56±0.28^{ab}	9.86±0.49^b	24.59±1.23^c
∑*n*-6/∑*n*-3	0.17±0.01^a	0.38±0.01^a	0.36±0.01^a	0.35±0.01^a	0.25±0.01^a	0.18±0.01^a	0.1±0.01^a	0.04±0^a
∑PUFA/∑SFA	16.47±0.82^a	4.39±1.07^b	3.96±1.24^b	4.79±1.98^b	19.11±4^a	180.02±9^a	322.27±16.11^c	182.02±9.1^d
∑TRANS	0.03±0^a	0.02±0^a	0.01±0^a	ND	ND	ND	ND	ND

On the basis of the significance of treatments LSD at the 5% level of significance ($p<0.05$) was computed. Data presented as mean values of three samples (mean ± standard deviation). ND) fatty acids identified on the GC trace but not integrated by the instrument. % Recovery is represented with respect to the fatty acid concentrate.

The fatty ester profiles of the various solvent fractions obtained by the silver-silica gel fractionation of fatty acid esters using diethyl ether:*n*-hexane as eluent are shown in Table 5.9.2. Because the SFAs (C14:0, C16:0) and the MUFAs (C16:1*n*-7, C18:1*n*-9) associate least strongly with the stationary phase, these esters are eluted quantitatively with the FD_1, FD_2 and FD_3 (i.e. the one containing 1, 3 and 5 % diethyl ether in *n*-hexane). The column fractions FD_4, FD_5, FD_6 and FD_7 showed significant increase in the ∑PUFA content (> 77 %) compared with initial value of 73.9 %. The fraction from 10% diethyl ether/*n*-hexane (FD_6) eluted EPA ester of higher purity (42.4 %) with 19.8 % total recovery. The highly unsaturated EPA (C20:5*n*-3) is barely eluted in the first four fractions (1-5% diethyl ether/*n*-hexane). Though DHA content showed substantially higher purity when eluted with 100% diethyl ether (FD_7 47 %) the total recovery was observed to be only 8.8 %. This is certainly because DHA is more hydrophobic relative to EPA and, therefore, has a higher affinity toward the relatively apolar eluent. However, a DHA content of 38.5 % was observed in 10 % diethyl ether/*n*-hexane (FD_6) with high recovery (Fig. 5.10). As shown in Table 5.9.2, the saturated fatty esters (C14:0, C16:0, C18:0) elute with the initial fractions (FD_{1-3}) because they do not complex with the Ag^+ ion. Similarly, the monounsaturated esters (C16:1*n*-7, C18:1*n*-9), that complex relatively less strongly with silver, are eluted mostly in the initial solvent fractions (FD_{1-3}). However, 31.6 % MUFAs eluted in the first fraction (FD_1) and in FD_5 the % MUFA was found to be significantly low compared to CO and FD_{1-4}. The PUFAs started eluting predominantly in FD_5 and its amount was found to be maximum in FD_7 (95.1 %) with 8.8 % total recovery. The elution using 100 % diethyl ether (FD_7) showed high percent of EPA and DHA (38.6 & 47 %, respectively) (Table 5.9.2; Fig. 5.10). It is to be noted that maximum EPA was observed in FD_5 (43 %) with 11.6 % total recovery. However, using *n*-hexane: acetone as eluent (FS_6) in Ag-silica column showed the dominance of EPA (47.7 %) with best total recovery (31.6 %). It is to be further noted that DHA, ∑PUFA, and ∑*n*-3PUFA also increased significantly higher in FS_6 compared to FD_5 demonstrating the importance of *n*-hexane: acetone as the solvent system for Ag-silica chromatographic purification of fatty acid concentrate.

Table 5.9.2 Recovery (% w/w) and fatty acid composition of the column fractions obtained by argentated silica column chromatography using diethylether/*n*-hexane as eluent

	CO	FD_1	FD_2	FD_3	FD_4	FD_5	FD_6	FD_7
Recovery		6.2	13.6	2.8	36.4	11.6	19.8	8.8
Saturated								
14:0	0.97±0.1^{a}	2.63±0.26^{a}	1.95±0.2^{a}	1.64±0.16^{a}	0.01±0^{a}	0.02±0^{a}	0.14±0.01^{a}	0.01±0^{a}
15:0	0.16±0.02^{a}	0.34±0.03^{a}	0.2±0.02^{a}	0.1±0.01^{a}	0.91±0.09^{a}	0.02±0^{a}	0.03±0^{a}	0.01±0^{a}
16:0	0.64±0.06^{a}	0.87±0.09^{a}	0.51±0.05^{a}	0.44±0.04^{a}	0.13±0.01^{a}	0.11±0.01^{a}	ND	0.01±0^{a}
17:0	0.47±0.05^{a}	0.52±0.05^{a}	0.44±0.04^{a}	0.38±0.04^{a}	0.21±0.02^{a}	0.06±0.01^{a}	0.03±0^{a}	0.04±0^{a}
18:0	1.94±0.19^{a}	3.21±0.31^{a}	2.51±0.25^{a}	2.26±0.23^{a}	1.38±0.13^{a}	0.37±0.04^{a}	0.25±0.03^{a}	0.13±0.01^{a}
20:0	0.1±0.01^{a}	0.26±0.03^{a}	0.1±0.01^{a}	0.45±0.05^{a}	0.07±0.01^{a}	0.08±0.01^{a}	ND	ND
22:0	0.08±0.01^{a}	0.1±0.01^{a}	0.06±0.01^{a}	0.5±0.05^{a}	0.07±0.01^{a}	0.02±0^{a}	0.03±0^{a}	0.01±0^{a}
24:0	0.13±0.01^{a}	0.15±0.02^{a}	0.07±0.01^{a}	0.12±0.01^{a}	0.14±0.01^{a}	0.06±0.01^{a}	0.14±0.01^{a}	0.12±0.01^{a}
∑SFA	4.49±0.45^{ab}	8.08±0.81^{a}	5.84±0.58^{ab}	5.89±0.59^{ab}	2.92±0.29^{b}	0.74±0.07^{b}	0.62±0.06^{b}	0.33±0.03^{b}
Monounsaturated								
14:1*n*-7	0.1±0.01^{a}	0.21±0.02^{a}	0.16±0.02^{a}	0.15±0.02^{a}	0.08±0.01^{a}	0.05±0.01^{a}	0.03±0^{a}	0.02±0^{a}
15:1*n*-7	0.06±0.01^{a}	0.11±0.01^{a}	0.06±0.01^{a}	0.03±0^{a}	0.05±0.01^{a}	0.02±0^{a}	0.01±0^{a}	0.01±0^{a}
16:1*n*-7 *trans*	0.02±0^{a}	ND	ND	ND	0.02±0^{a}	ND	ND	ND
16:1*n*-7	4.8±0.48^{a}	14.56±0.45^{b}	11.39±0.14^{b}	10.69±0.07^{bc}	6.23±0.61^{ac}	1.61±0.16^{a}	1.04±0.1^{a}	0.59±0.06^{a}
18:1*n*-9 *trans*	0.01±0^{a}	ND	ND	ND	ND	ND	ND	ND
18:1*n*-9	5.74±0.57^{ab}	9.32±0.92^{a}	7.31±0.73^{a}	6.7±0.67^{ab}	3.78±0.38^{b}	1.46±0.15^{b}	0.62±0.06^{b}	0.4±0.04^{b}
20:1*n*-9	0.04±0^{a}	1.17±0.12^{a}	0.04±0^{a}	0.97±0.1^{a}	0.2±0.02^{a}	0.02±0^{a}	0.14±0.01^{a}	0.01±0^{a}
22:1*n*-9	4.34±0.43^{a}	5.96±0.6^{a}	4.93±0.49^{a}	4.62±0.46^{a}	3.86±0.39^{a}	0.27±0.03^{a}	0.21±0.02^{a}	0.13±0.01^{a}
24:1*n*-9	0.38±0.04^{a}	0.23±0.02^{a}	0.29±0.03^{a}	0.44±0.04^{a}	0.63±0.06^{a}	0.16±0.02^{a}	0.31±0.03^{a}	0.12±0.01^{a}
∑MUFA	15.49±0.55^{a}	31.56±0.16^{b}	24.18±0.42^{c}	23.6±0.36^{c}	14.85±0.49^{a}	3.59±0.36^{d}	2.36±0.24^{d}	1.28±0.13^{d}
Polyunsaturated								
18:2*n*-6 *trans*	ND	0.01±0^{a}	ND	ND	0.01±0^{a}	ND	ND	ND
18:2*n*-6	3.09±0.31	3.97±0.4^{a}	4.12±0.41	3.96±0.4	4.23±0.42	4.58±0.46	2.61±0.26	1.64±0.16
18:3*n*-6	0.59±0.06^{a}	0.4±0.04^{a}	1.05±0.11^{a}	0.28±0.03^{a}	0.65±0.07^{a}	0.25±0.03^{a}	0.04±0^{a}	0.04±0^{a}
18:3*n*-3	0.04±0^{a}	0.08±0.01^{a}	0.22±0.02^{a}	0.03±0^{a}	0.7±0.07^{a}	0.06±0.01^{a}	0.01±0^{a}	0.01±0^{a}
20:2*n*-6	5.9±0.59^{a}	4.83±0.48^{a}	5.29±0.53^{a}	5.19±0.52^{a}	5.46±0.54^{a}	4.56±0.46^{a}	2.36±0.24^{a}	1.35±0.14^{a}
20:3*n*-6	0.04±0^{a}	0.2±0.02^{a}	0.09±0.01^{a}	0.09±0.01^{a}	0.06±0.01^{a}	3.07±0.31^{a}	1.3±0.13^{a}	0.73±0.07^{a}
20:4*n*-6	1.12±0.11^{a}	1.26±0.13^{a}	1.19±0.12^{a}	1.14±0.11^{a}	0.99±0.01^{a}	0.73±0.07^{a}	0.39±0.04^{a}	0.26±0.03^{a}
20:5*n*-3 EPA	33.37±3.34^{ac}	24.01±0.4^{b}	26.33±0.63^{b}	28.24±0.82^{ab}	31.51±0.15^{a}	42.93±0.29^{c}	42.36±0.24^{c}	38.65±0.87^{c}
22:5*n*-3	2.03±0.2^{a}	3.33±0.33^{a}	3.08±0.31^{a}	1.89±0.19^{a}	2.75±0.28^{a}	3.08±0.31^{a}	6.07±0.61^{a}	5.47±0.54^{a}
22:6*n*-3 DHA	27.76±0.78^{ac}	18.73±0.87^{b}	25.73±0.57^{ac}	26.86±0.69^{ac}	31.32±0.13^{c}	34.74±0.47^{c}	38.47±0.84^{c}	46.97±0.07^{d}
∑PUFA	73.94±0.39^{a}	56.82±0.68^{b}	67.1±0.71^{c}	67.68±0.77^{c}	77.68±0.77^{d}	94±0.04^{e}	93.61±0.36^{e}	95.12±0.51^{e}
EPA+DHA	61.13±0.11^{a}	42.74±0.27^{b}	52.06±0.21^{c}	55.1±0.51^{c}	62.83±0.28^{a}	77.67±0.07^{d}	80.83±0.08^{d}	85.62±0.56^{d}
∑*n*-3PUFA	63.2±6.02^{a}	46.15±0.62^{b}	55.36±0.54^{c}	57.02±0.07^{a}	66.28±0.63^{a}	80.81±0.08^{d}	86.91±0.69^{d}	91.1±0.01^{d}
∑n-6PUFA	10.74±0.07^{a}	10.66±0.07^{a}	11.74±0.17^{a}	10.66±0.07^{a}	11.39±0.14^{a}	13.19±0.32^{a}	6.7±0.67^{b}	4.02±0.4^{b}
∑*n*-3/∑*n*-6	5.88±0.59^{a}	4.33±0.03^{a}	4.72±0.47^{a}	5.35±0.54^{a}	5.82±0.08^{a}	6.13±0.61^{a}	12.97±0.3^{b}	22.66±0.07^{e}
∑*n*-6/∑*n*-3	0.17±0.02^{a}	0.23±0.02^{a}	0.21±0.02^{a}	0.19±0.02^{a}	0.17±0.02^{a}	0.16±0.02^{a}	0.08±0.01^{a}	0.04±0^{a}
∑PUFA/∑SFA	16.47±0.65^{a}	7.03±0.7^{b}	11.49±0.15^{ab}	11.49±0.15^{ab}	26.6±0.66^{c}	127.03±0.07^{d}	150.98±0.01^{e}	288.24±0.05^{f}
∑TRANS	0.03±0^{a}	0.01±0^{a}	ND	ND	0.03±0^{a}	ND	ND	ND

On the basis of the significance of treatments LSD at the 5% level of significance (p) 0.05) was computed. Data presented as mean values of three samples (mean ± standard deviation). ND fatty acids identified on the GC trace but not integrated by the instrument. % Recovery is represented with respect to the fatty acid concentrate.

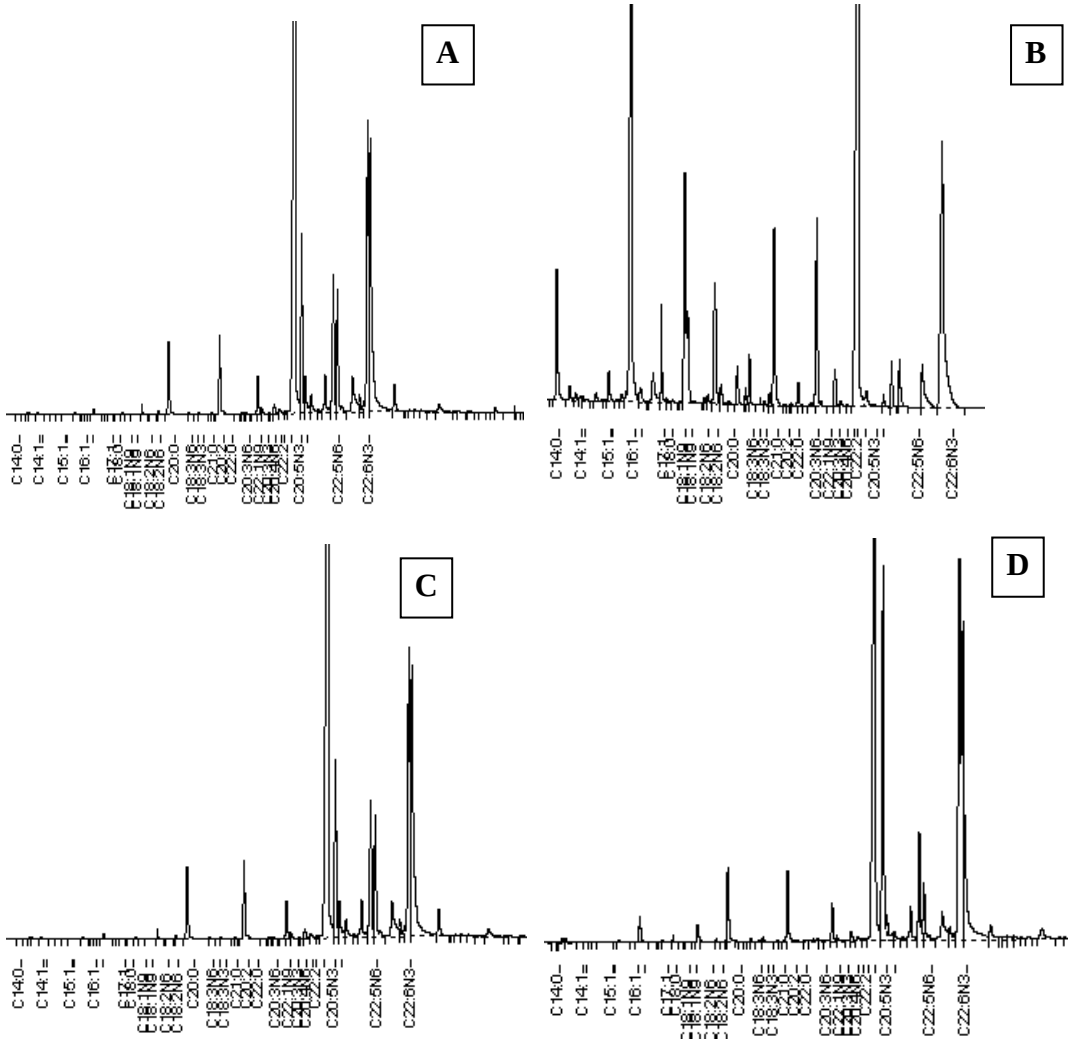


Fig. 5.10. Fatty acid chromatogram of (A) FA_6; (B) FS_2; (C) FS_6 and (D) FD_6

5.2.2.3.1C. Argentation Chromatography using Ag-silica stirring

The fatty ester profiles of the various solvent fractions obtained by stirring with $AgNO_3$-silica using *n*-hexane, *n*-hexane: Me_2O (1:1) and Me_2O are shown in Table 5.9.3. It is to be note that ∑MUFA significantly increased in all the fractions obtained by stirring with *n*-hexane (FT_1), *n*-hexane: Me_2O (1:1) (FT_2) and Me_2O (FT_3) (29.7, 26.6 & 20.8 %, respectively) ($p<0.05$). However, though insignificant, a reduction in ∑PUFA, EPA, DHA was observed for FT_1, FT_2 and FT_3. This proved that the stirring was not effective in the purification of PUFAs compared to the column chromatographic techniques.

Table 5.9.3 Recovery (% w/w) and fatty acid composition of the fractions obtained by stirring with Ag-silica

	CO	FT_1	FT_2	FT_3
Recovery		24.2	35.4	29
Saturated				
14:0	0.97±0.1[a]	2.76±0.28[a]	2.2±0.22[a]	1.75±0.18[a]
15:0	0.16±0.02[a]	0.13±0.01[a]	0.09±0.01[a]	0.08±0.01[a]
16:0	0.64±0.06[a]	0.6±0.05[a]	0.51±0.05[a]	0.44±0.04[a]
17:0	0.47±0.05[a]	0.48±0.05[a]	0.4±0.04[a]	0.33±0.03[a]
18:0	1.94±0.19[a]	2.62±0.26[a]	2.18±0.22[a]	1.77±0.17[a]
20:0	0.1±0.01[a]	0.1±0.01[a]	0.1±0.01[a]	0.07±0.01[a]
22:0	0.08±0.01[a]	0.08±0.01[a]	0.08±0.01[a]	0.07±0.01[a]
24:0	0.13±0.01[a]	0.09±0.01[a]	0.12±0.01[a]	0.1±0.01[a]
∑SFA	4.49±0.44[a]	6.86±0.69[a]	5.68±0.57[a]	4.61±0.46[a]
Monounsaturated				
14:1*n*-7	0.1±0.01[a]	0.15±0.02[a]	0.11±0.01[a]	0.11±0.01[a]
15:1*n*-7	0.06±0.01[a]	0.03±0[a]	0.03±0[a]	0.02±0[a]
16:1*n*-7 *trans*	0.02±0[a]	ND	ND	ND
16:1*n*-7	4.8±0.48[a]	13.99±0.04[b]	12.9±0.29[b]	9.56±0.96[b]
18:1*n*-9 *trans*	0.01±0[a]	ND	ND	ND
18:1*n*-9	5.74±0.57[a]	9.32±0.93[a]	8.09±0.81[a]	5.93±0.59[a]
20:1*n*-9	0.04±0[a]	0.04±0[a]	0.03±0[a]	0.8±0.08[a]
22:1*n*-9	4.34±0.43[a]	4.81±0.48[a]	4.53±0.45[a]	4.08±0.41[a]
24:1*n*-9	0.38±0.04[a]	1.37±0.14[a]	0.88±0.09[a]	0.32±0.03[a]
∑MUFA	15.49±0.05[ac]	29.71±0.07[b]	26.57±0.66[b]	20.82±0.08[c]
Polyunsaturated				
18:2*n*-6 *trans*	ND	0.01±0[a]	0.01±0[a]	ND
18:2*n*-6	3.09±0.31[a]	4.16±0.42[a]	3.76±0.38[a]	3.38±0.34[a]
18:3*n*-6	0.59±0.06[a]	1.1±0.11[a]	0.96±0.1[a]	0.25±0.03[a]
18:3*n*-3	0.04±0[a]	0.08±0.01[a]	0.09±0.01[a]	0.07±0.01[a]
20:2*n*-6	5.9±0.59[a]	4.98±0.5[a]	4.79±0.48[a]	4.55±0.45[a]
20:3*n*-6	0.04±0[a]	0.16±0.02[a]	0.15±0.02[a]	0.16±0.02[a]
20:4*n*-6	1.12±0.11[a]	1.16±0.12[a]	0.25±0.03[a]	0.48±0.05[a]
20:5*n*-3 EPA	33.37±0.32[a]	23.5±0.34[b]	25.85±0.57[b]	30.83±0.07[a]
22:5*n*-3	2.03±0.02[a]	1.7±0.07[a]	2.35±0.04[a]	3.31±0.33[a]
22:6*n*-3 DHA	27.76±0.77[a]	19.19±0.92[b]	23.01±0.3[ab]	26.3±0.63[a]
∑PUFA	73.94±0.39[a]	56.04±0.6[b]	61.22±0.12[bc]	69.33±0.93[ac]
EPA+DHA	61.13±0.11[a]	42.69±0.27[b]	48.86±0.89[bc]	57.13±0.71[ac]
∑*n*-3PUFA	63.2±0.32[a]	44.47±0.45[b]	51.3±0.13[bc]	60.51±0.05[ac]
∑n-6PUFA	10.74±0.07[a]	11.56±0.16[a]	9.91±0.09[a]	8.82±0.88[a]
∑*n*-3/∑*n*-6	5.88±0.59[a]	3.85±0.09[a]	5.18±0.52[a]	6.86±0.69[a]
∑*n*-6/∑*n*-3	0.17±0.02[a]	0.26±0.03[a]	0.19±0.02[a]	0.15±0.02[a]
∑PUFA/∑SFA	16.47±0.65[a]	8.17±0.82[b]	10.78±0.08[b]	15.04±0.5[a]
∑TRANS	0.03±0[a]	0.01±0[a]	0.01±0[a]	ND

On the basis of the significance of treatments LSD at the 5% level of significance (p) 0.05) was computed. Data presented as mean values of three samples (mean ± standard deviation). ND - fatty acids identified on the GC trace but not integrated by the instrument. % Recovery is represented with respect to the fatty acid concentrate.

5.2.2.3.1D. Spectral Analyses of Purified Methyl Esters (FS_6)

Purification of fatty acid methyl esters was performed as these compounds exhibit more stability and better chromatographic behavior than free fatty acids.

Normal pressure column chromatography on silica gel impregnated with $AgNO_3$ was used to separate different fatty acids according to the differences in their degree of unsaturation. The resolving power of Ag-silica is largely attributed to a reversible charge-transfer complexation of Ag^+ ion with -C=C- double bonds of unsaturated compounds. The stability of the complex increases with an increasing number of double bonds but decreases with the increasing chain length. The extent and the strength of complexation control the mobility of a solute (fatty acids), as does the polarity of the mobile phase. The separation achieved by argentation column chromatography was continuously monitored by $AgNO_3$TLC and GC/GC-MS.The process of argentation is applied to remove the saturated and monounsaturated fatty acid methyl esters.

The process of argentation is applied to remove the saturated and monounsaturated fatty acid methyl esters. The quality of the concentrated methyl ester fraction eluted with 10 % acetone: *n*-hexane on Ag-silica (FS_6) was for ^{1}H-NMR spectroscopy (Fig. 5.11A) conjugated with ^{13}C-NMR (Fig. 5.11B) methodology which applies DEPT (Fig. 5.11C) pulse sequence. The olefinic protons –CH=CH– of PUFAs resonate at δ 5.3–5.5 ppm (m), the multiplet is not baseline resolved from the proton signal at δ 5.33 ppm (m). ^{1}H-NMR spectrum demonstrated the presence of sharp signal at δ 3.56 ppm due to the protons associated with the methyl ester (-C(=O)OCH_3) group. The higher proton integral of the *bis*-allylic peaks at 3 ppm also indicated the predominance of polyunsaturated fatty acid content in the winterized oil. The proton integral too demonstrated less signals at δ 1.2-1.5 ppm which apparently indicated the low content of long hydrocarbon moiety of the SFAs. These observations have been corroborated with the ^{13}C-NMR/$DEPT_{135}$ and infra-red analyses data.

The characteristic >C=O signal appeared at δ 180 ppm in the ^{13}C-NMR spectrum, whereas the olefinic protons showed peaks at about δ 125 - 135 ppm. The -CH_2- and -CH_3 carbons of FS_6 appeared at δ 15 - 35 ppm, whereas the -CH_2- group interrupting the two double bonds appeared downfield at δ 38 ppm apparently due to the electron withdrawing effects of the olefinic system. The appearance of downfield carbons at δ 40 ppm also demonstrated the fact that the carbon is located in close

vicinity of a >C=O group (-COOMe) and an olefinic double bond, apparently from long chain polyunsaturated fatty acids such as DHA and EPA. The resonances of allylic and *bis*–allylic methylenes were reported except for the methylene envelope – $(CH_2)_n$, which was indicated at δ 30 ppm as a whole. These results have been corroborated by the fatty acid analyses of FS_6.

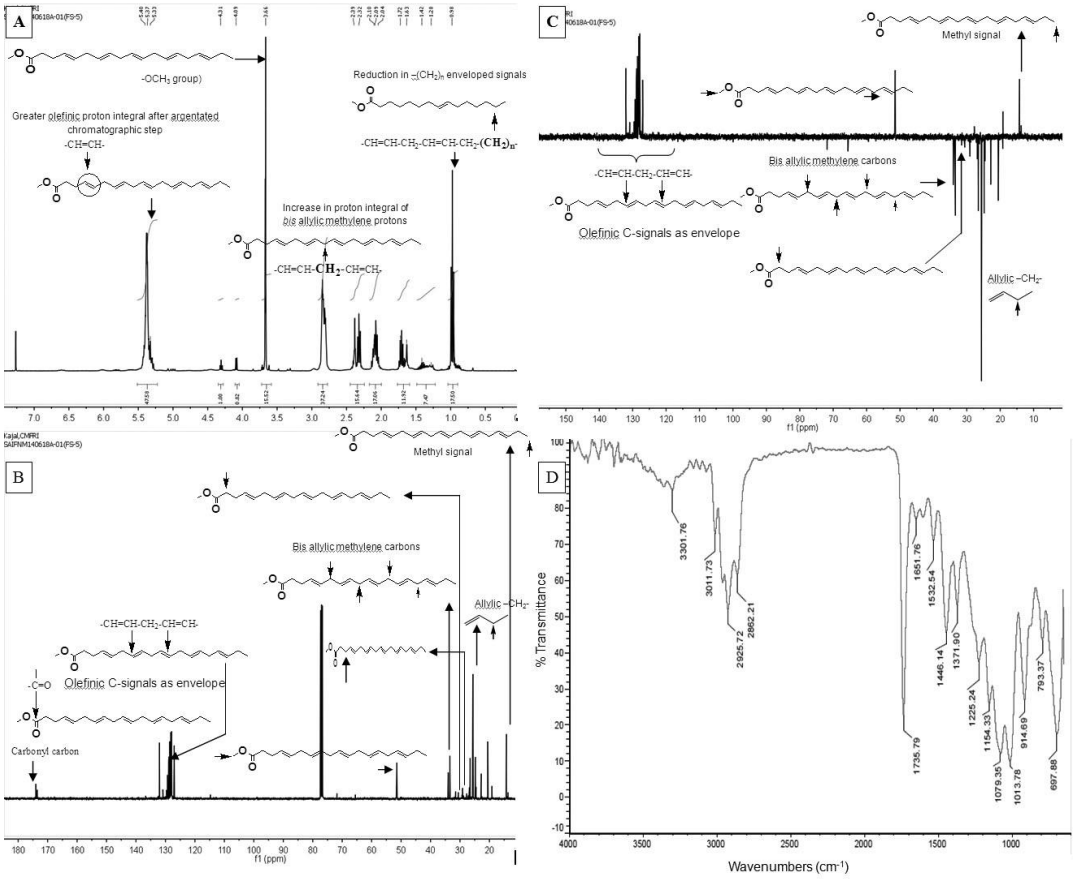


Fig. 2

Fig. 5.11. (A) ^{1}H, (**B**) ^{13}C, (**C**) $DEPT_{135}$ and (**D**) FTIR spectra of purified fatty acid methyl esters FS_6.

The methyl esters FS_6 obtained by eluting with 10 % acetone:*n*-hexane using Ag-silica was further validated using FT-IR analysis (Fig. 5.11D). FT-IR spectra of FS_6 revealed a significant reduction in C-H stretching vibrations (at 2800-3000 cm^{-1}), and bending vibrations at the IR fingerprint region (400-1000 cm^{-1}) as compared to that in crude sardine oil, thereby explaining the absence of hydrocarbon impurities. However, an increased absorption of the olefinic region (1079, 1013 cm^{-}

^{1}) was apparent in the FTIR spectrum of FS_6. It is also interesting to note that the C-H stretching vibration was found to be slightly greater than 3000 cm^{-1}, which led us to infer that it is only possible for alkenes to show a C-H stretch slightly greater than 3000 cm^{-1}. Compounds that do not have a C=C bond show C-H stretches only below 3000 cm^{-1}. To state otherwise, the PUFAs were significantly enriched in ~~**CME**~~ $CMES_6$ obtained by the process of argentated silica column chromatography. However, the strongest bands in the FTIR spectrum were those attributed to the C-H bending vibrations of the =C–H group in the region 1000-650 cm^{-1}, which apparently overlap the fingerprint region. The stretching vibration of the C=C bond exhibited a moderate band in the region 1680-1651 cm^{-1}.

5.2.3.Preparation of Purified Essential Fatty Acids using Biotechnological, Physical and Chemical Techniques

5.2.3.1. Isolation, Production, Purification and Molecular Weight Determination of Lipase from *Bacillus circulans*

5.2.3.1A. Preparation of Crude Lipase from Bacillus circulans

Lipase-selective medium (rhodamine B-triolein agar) was used to determine the lipase-producing capability of *B. circulans* isolated from the marine macroalga *T. conoides*. The crude extracts obtained after 24 h of incubation exhibited a lipase activity of 7.3 ± 0.8 LU/ ml. However, the crude extract obtained after 34 h of incubation recorded the highest lipase activity (12.2 ± 1.1 LU/ml) (Fig. 5.12 A-C), and was therefore used for further purification of the lipase. Earlier studies indicated maximum lipase activities at the preset of logarithmic phase of bacterial growth (Schuepp *et al.*, 1997).

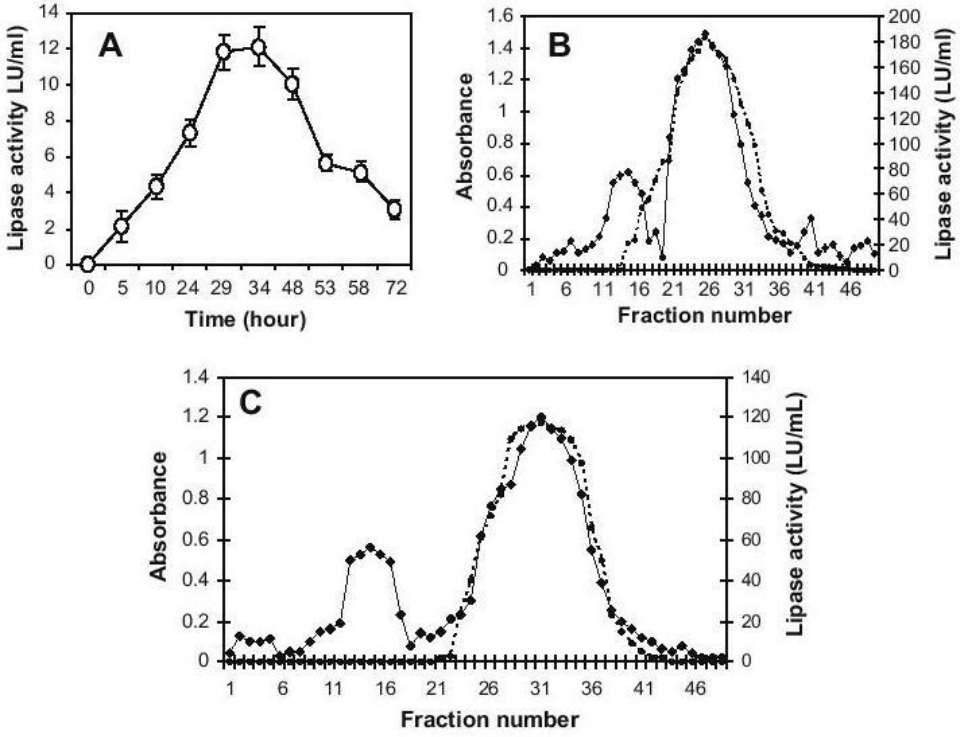


Fig. 5.12. (**A**) Time course-dependent lipase activity of a culture of *B. circulans*. Extracellular activity of lipase was assayed with 4-nitrophenyl palmitate. (**B**) Chromatographic purification profile of the lipase on Sephadex G-100 gel exclusion column, and (**C**) anion exchange column (Amberlite IRA-410 Cl^- form).

5.2.3.1B. Purification of B. Circulans Lipase and Molecular Studies

The results of purification profile of the extracellular lipase by *B. circulans* are summarized in Table 5.10. A 109-fold purification with a specific activity of 119 LU/mg was attained after gel filtration on sephadex G-100 (Table 5.10). Highest lipase activity was apparent at the 26^{th} fraction of the gel filtration (Fig. 5.13). The enzyme was finally purified 132-fold, using amberlite XRD-5 (Cl^- form) anion- exchange chromatography, at the 32^{nd} fraction, with a specific activity of 386 LU/mg (Table 5.10). The homogeneity of the purified lipase was checked by the presence of a single band corresponding to an apparent molecular mass of 39.8 kDa on SDS–PAGE gels, suggesting it to be a homomeric protein (Fig. 5.13). Activity staining confirmed the presence of the purified lipase. Gel filtration chromatography, using Sephadex G-75, indicated the apparent molecular weight of the lipase as 40.5 kDa. (Fig. 5.13)

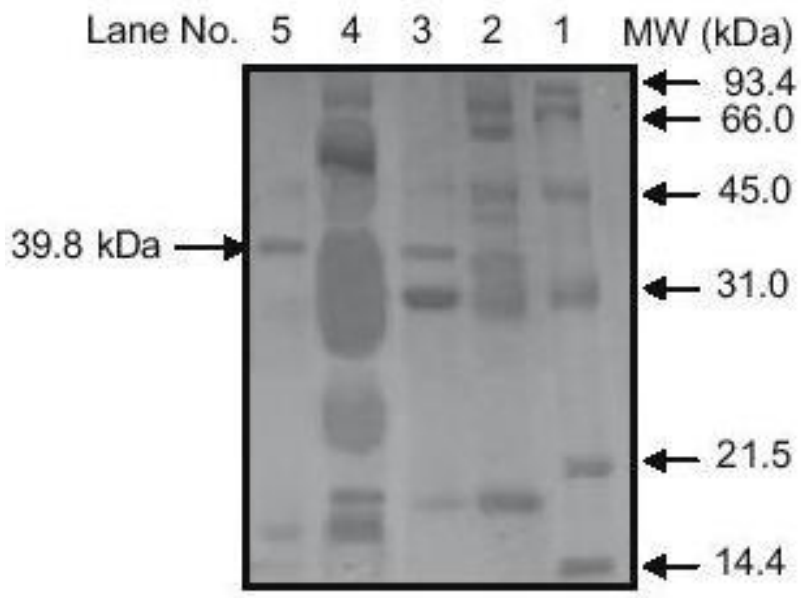


Fig. 5.13. Denaturing polyacrylamide gel electrophoretic analyses of lipase obtained at different stages of purification from culture broth of B. circulans on 12% polyacrylamide gel. Lane 1: low-range molecular mass markers (Bio-Rad Laboratories, Hercules, CA): phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa); lane 2: 20 lg of total proteins after ammonium sulphate precipitation; lane 3: 20 lg of purified lipase after Sephadex G-100 gel filtration; lane 4: 20 lg of crude protein; lane 5: 20 lg of total proteins after Amberlite IRA- 410 (Cl^- form) anion-exchange chromatography. The purified lipase was represented by a single band corresponding to a molecular mass of about 39.8 kDa.

Table 5.10 Summary of various steps involved in the lipase purification scheme from *B. circulans* culture broth (34 h, 37 °C)

	Total activity (LU)[c]	Total protein (mg)[d]	Specific activity (LU/mg) Yield	Yield (%)	Purification (fold)
Crude culture broth (34 h, 37 °C)[a]	6074±116	2080±129	2.9	100	1
$(NH4)_2SO_4$ precipitation[b]	3605±118	320±18.7	11.3	46.7	3.9
Gel filtration chromatography	1839±103	15.4±1.8	119	12.4	109
Anion-exchange chromatography	1173±96.5	3.0±0.2	386	5.2	132

[a] *Bacillus circulans* was grown in selective broth, and the harvested broth was clarified to obtain a supernatant that was termed crude lipase.

[b] The crude lipase was purified by ammonium sulphate salt precipitation, Amberlite IRA-410 (Cl^- form) anion-exchange, and Sephadex G-100 gel filtration chromatography.

[c] The lipase activity (LU) was assayed by a spectrophotometric method, by monitoring 4-nitrophenyl palmitate hydrolysis, as detailed in *Section 5.1.4.1B*.

[d] Total protein was estimated by Bradford method. Crude culture broth was taken to a total volume of 500 ml.

5.2.3.2. Change in Fatty Acid Composition as a Function of Time-Course (1-6 h) Lipase-Catalyzed Hydrolysis of Refined Oil

The refined oil (RO) was found to contain PUFAs, particularly 18:2*n*-6 (2 %), 20:2*n*-6 (1.3 %), 20:5*n*-3 (15.5 %) and 22:6*n*-3 (5 %), along with other *n*-3 and s, as indicated in Table 5.11. The *n*- 3 fatty acids contributed the major share (22.1 %) with the *n*-3/*n*-6 fatty acid ratio of 5.1. Amongst saturated fatty acids, 16:0 was found to be predominant, followed by 14:0, and 18:0 (Table 5.11). The RO was hydrolyzed with lipases purified from *B. circulans*, the total fatty acid content at various time-intervals of hydrolysis (1 - 6 h) were analyzed, and the results are indicated in Table 5.11. The lipase produced the highest degree of hydrolysis for SFAs (18.7 %), followed by MUFAs (25.7 %), from their initial content after 3 h. The total concentration of SFA exhibited reductions of 27.5 % and 54.4 % after 1 and 3 h of hydrolysis, respectively whereas, after prolonged hydrolysis (6 h), the proportion of SFAs to total fatty acids was found to be increased to 21.8 % (Table 5.11). The total MUFA content was found to be reduced by 12 % after 3 h of lipase-catalyzed hydrolysis and 17.2 % after 6 h. The decrease in the contents of SFAs and MUFAs with the progress of hydrolysis suggests that SFAs and MUFAs were more easily hydrolyzed by the lipase than those in triglycerides that contain PUFAs, resulting in their enrichment in the triglyceride fraction. It was reported that those substrates containing D_2–D_7 isomers of 18:1*n*-9 were resistant to lipase-catalyzed hydrolysis, resulting in higher concentration of the fatty acid, and the discrimination was greatest for the D_5 isomer (Heimermann *et al.,* 1973). The reduction in SFAs and MUFAs in the enzyme hydrolysate appeared to be due to the higher selectivity of lipase for SFAs and MUFAs, thus furnishing a fatty acid concentrate with comparatively lower content of these fatty acids (Chakraborty & Paulraj, 2008).

The lipase was able to enrich the fatty acids with 37.7 % of 20:5*n*-3 after 3 h of hydrolysis of triglycerides, which was significantly higher than that in RO (Table 5.11) ($p<0.05$). The total *n*-3 PUFAs increased with time up to 3 h of lipase-catalyzed hydrolysis (50.4 %), beyond which it plateaued to 47.2 % at 6 h, apparently due to the reduced selectivity of the lipase. Other *n*-3 PUFAs with a C_{18}

acyl side chain, e.g. 18:3*n*-3 and 18:4*n*-3, also exhibited an increasing trend in their concentration after 1 and 3 h of lipase-catalyzed hydrolysis (Table 5.11). After 3 h, these fatty acids were recorded as 5.1 % and 2.3 %, respectively, and significantly higher than the initial content ($p<0.05$). However, with increase of acyl chain length (>C20), hydrolytic susceptibility of the ester linkage of triglycerides (by the lipase) was found to increase as is evident from the less increment of 12.1% decrease of 22:6*n*-3 in the triglyceride fraction after 3 h of hydrolysis. The final 22:6*n*-3 content in the glyceride mixture after 6 h hydrolysis was 6.5 %, i.e., 1.3 -fold increase from RO (Table 5.11). Similar results have been apparent from the reduced increment of 22:5*n*-3. Generally lipases are very specific in hydrolyzing specific fatty acids with specific acyl chain length (e.g. C_{20} or C_{22} acyl chain length) or specific classes of fatty acids (e.g. EPA with C_{20} acyl chain length and DHA C_{22} acyl chain length). Since lipase reaction is very specific, some lipases (e.g. the *B. circulans* lipase in this study) can concentrate a particular fatty acid (e.g. EPA, 20:5*n*-3), but may be ineffective for concentrating other fatty acids (e.g. DHA 22:6*n*-3, which could not be enriched in this process). The bacterial lipase purified from *B. circulans* is specific in hydrolyzing fatty acids higher than C20 *n*-3, e.g. DHA, thereby decreasing their concentration in the triglyceride fraction. A 2-fold increase of DHA and EPA concentration was reported by *Pseudomonas cepacia* and *Candida rugosa* lipase catalyzed hydrolysis of *Salmo salar* L. oil (Sun *et al.,* 2002). A microbial lipase isolated from *C. antarctica* was reported to yield 93.5 % PUFA with 25.7 % EPA and 44.7 % DHA from cod liver oil triglycerides (Medina *et al.,* 1999). Hydrolysis by *P. fluorescens* HU380 lipase, followed by amide complexation of cod liver oil, was reported to yield 43.1% EPA and 7% DHA (Kojima *et al.,* 2006). From these results it can be concluded that the bacterial lipase is specific in hydrolyzing fatty acids higher than C20:*n*-3. It is apparent that a particular fatty acid, e.g. 22:5*n*-3 or 22:6*n*-3, released on hydrolysis is more abundant, resulting in decrease of the triacylglycerol portion of fatty acids, thus suggesting that the ester bond of the fatty acids are susceptible to hydrolysis by the lipase used in the present study. On the other hand, 20:5*n*-3 and 18:3*n*-3 (having the C18 *n*-3 configuration) are more abundant in the triacylglycerol portion of fatty acids, thus suggesting that the lipase

is less reactive towards that ester bond of fatty acids (resistant to hydrolysis), resulting in their increase in the triglycerides (Chakraborty & Paulraj 2008). However, due to its acyl side chain and olefinic double bond specificity, this particular lipase obtained from *B. circulans* is not able to enhance the DHA content. In general, the *n*-6 fatty acids exhibited a reduction in their content after lipase-catalyzed hydrolysis. Amongst *n*-6 PUFAs, 18:2*n*-6 was recorded to be 0.04% after 1 h of lipase-catalyzed hydrolysis, and 0.4 % after 3 h, which were substantially lower than that in RO (Table 5.11). Similarly, the content of 20:4*n*-6 in triglyceride was found to decrease by 81.5 % after 1 h of hydrolysis (Table 5.11). The results apparently suggest that, amongst *n*-3 and *n*-6 fatty acids, the former are more resistant to hydrolysis by the lipase used in this study, and the discrimination was the greatest for the $C_{18–20}$ acyl chain-lengthened homologues. An earlier study reported that the content of 20:4*n*-6 in the glyceride mixture increased with the progress of hydrolysis by lipase purified from *B. licheniformis* (Chakraborty & Paulraj 2008). This was found to be due to fatty acid specificity of lipases toward PUFAs with Δ^5 unsaturated double bond (*cis*-5 olefinic bond positioned at the number 5 carbon from the carboxyl end) (Chakraborty & Paulraj 2008). Earlier results indicated that the lipase from *B. licheniformis* was unique in the resistance it encountered with 20:5*n*-3 and 20:4*n*-6. This could be attributed to the positional specificity of the enzyme at the olefinic double bonds of PUFAs. Lipases have been screened, from different sources, to selectively concentrate α-linolenic acid from *B. officinalis* and *Echium fastuosum* seed oil (López-Martínez *et al.*, 2004).

5.2.3.3. Concentration and Purification of Lipase Hydrolyzed Sardine Fatty Acids (LHRF)

The lipase hydrolyzed sardine fatty acids (LHRF) was purified by urea complexation (at 4 ^{o}C using 4:1 ratio of urea: LHRF for 12 h contact time) to get lipase hydrolyzed concentrated sardine fatty acids (LHCO) and argentated-silica column chromatographic fractionation (using Me_2O:*n*-hexane) to obtain lipase hydrolyzed purified sardine oil (LHPO) as detailed below. The per cent fatty acid

composition obtained after urea complexation is illustrated in Table 5.11. Urea complexation resulted in the reduction in SFAs (2.6 %) and MUFAs (9.6 %) (Table5.11). The ∑PUFAs after urea complexation was recorded to be 86.7 %, mainly contributed by 20:5*n*-3 (51.3 %), 22:6*n*-3 (28 %), *n*-3 PUFA (83.4 %) and *n*-6 PUFA (3.3 %). In an earlier study, the fatty acids containing enriched 20:5*n*-3 (46.3%) and 20:4*n*-6 (3.9%) were recovered by partial hydrolysis using lipase purified from *B. licheniformis*, and were further concentrated by urea complexation to 55.4 and 5.8%, respectively (Chakraborty & Paulraj 2008). The percent fatty acid composition obtained after argentation chromatography of LHPO showed an increase of ∑PUFA upto 93.5 %, 20:5*n*-3 upto 60 %, 22:6*n*-3 upto 30 %, *n*-3 PUFA upto 92 % and *n*-6 PUFA upto 1.5 %.

Table 5.11 Fatty acid composition of refined sardine oil, lipase hydrolysate at three different time durations (1, 3, and 6 h) using purified lipase obtained from *B. circulans*, followed by urea concentrated sardine fatty acids (LHCO) and argentated silica column purified sardine methyl esters (LHPO)

Fatty acids	Refined Sardine Oil (RO)	1h	3h	6h	LHCO	LHPO
14:0	12.95±0.3^{a}	8.3±0.83^{ab}	5±0.5^{b}	7.4±0.74^{ab}	1.7±0.17^{c}	1.5±0.14^{c}
15:0	0.76±0.08^{a}	0.76±0.01^{a}	0.73±0.07^{a}	0.74±0.07^{a}	0.02±0^{a}	ND
16:0	21.98±0.1^{a}	17.1±0.7^{a}	9.3±0.93^{b}	11.1±0.1^{b}	0.7±0.07^{c}	0.5±0.05^{c}
17:0	0.8±0.08^{a}	0.84±0.08^{a}	0.87±0.09^{a}	0.09±0.01^{a}	0.01±0^{a}	0.01±0^{a}
18:0	4.13±0.01^{a}	2.2±0.22^{a}	2.4±0.24^{a}	2.1±0.21^{a}	0.01±0^{a}	0.01±0^{a}
20:0	0.42±0.04^{a}	0.31±0.03^{a}	0.12±0.01^{a}	0.11±0.01^{a}	0.02±0^{a}	ND
22:0	0.26±0.03^{a}	0.21±0.02^{a}	0.25±0.03^{a}	0.26±0.03^{a}	0.02±0^{a}	0.01±0^{a}
24:0	0.03±0^{a}	0.01±0^{a}	0.02±0^{a}	0.01±0^{a}	0.1±0.01^{a}	0.25±0.03^{a}
∑SFA	41.34±0.03^{a}	29.73±0.97^{b}	18.69±0.87^{c}	21.81±0.18^{bc}	2.58±0.26^{d}	2.28±0.23^{d}
14:1*n*-7	0.16±0.02^{a}	0.02±0^{a}	0.01±0^{a}	0.02±0^{a}	ND	0.01±0^{a}
15:1*n*-7	0.08±0.01^{a}	0.11±0.01^{a}	0.02±0^{a}	0.01±0^{a}	ND	0.02±0^{a}
16:1*n*-7 *trans*	0.04±0^{a}	0.02±0^{a}	0.01±0^{a}	0.03±0^{a}	ND	ND
16:1*n*-7	13.86±0.39^{ab}	15.4±0.54^{a}	10.32±0.03^{ab}	7.54±0.74^{b}	2.1±0.21^{c}	1.55±0.15^{c}
18:1*n*-9 *trans*	0.02±0^{a}	0.05±0.01^{a}	0.01±0^{a}	0.02±0^{a}	ND 0	ND
18:1*n*-9	9.69±0.07^{ab}	16.52±0.65^{b}	15.01±0.05^{b}	16.25±0.63^{b}	7.54±0.05^{a}	2.01±0.02^{c}
20:1*n*-9	0.04±0^{a}	0.3±0.03^{a}	0.2±0.02^{a}	0.2±0.02^{a}	ND	0.1±0.01^{a}
22:1*n*-9	2.47±0.05^{a}	0.1±0.01^{a}	ND	ND	ND	ND
24:1*n*-9	0.51±0.05^{a}	0.12±0.01^{a}	0.11±0.01^{a}	0.11±0.01^{a}	ND	ND
∑MUFA	26.88±1.69^{ac}	32.64±0.26^{a}	25.69±0.57^{bc}	24.18±0.42^{bc}	9.64±0.96^{d}	3.69±0.37^{d}
18:2*n*-6 *trans*	0.05±0.01^{a}	0.05±0.01^{a}	0.02±0^{a}	0.03±0^{a}	0.02±0^{a}	0.01±0^{a}
18:2*n*-6	1.93±0.19^{a}	0.04±0^{a}	0.4±0.04^{a}	0.4±0.04^{a}	1.6±0.16^{a}	0.3±0.03^{a}
18:3*n*-6	0.61±0.06^{a}	0.64±0.06^{a}	0.06±0.01^{a}	0.04±0^{a}	0.01±0^{a}	0.02±0^{a}
18:3*n*-3	0.26±0.03^{a}	0.21±0.02^{a}	5.1±0.01^{a}	3.2±0.03^{a}	2.7±0.26^{a}	2.01±0.2^{a}
20:2*n*-6	1.22±0.11^{a}	1.3±0.13^{a}	2.3±0.23^{a}	2.3±0.23^{a}	0.7±0.07^{a}	1.02±0.1^{a}
20:3*n*-6	0.3±0.03^{a}	0.32±0.03^{a}	0.02±0^{a}	0.02±0^{a}	0.05±0.01^{a}	0.04±0^{a}
20:4*n*-6	0.44±0.04^{a}	0.1±0.01^{a}	0.1±0.01^{a}	0.1±0.01^{a}	0.9±0.09^{a}	0.1±0.01^{a}

20:5*n*-3 EPA	12.85±0.29^{a}	23±0.3^{b}	37.7±0.77^{c}	37.1±0.71^{c}	51.3±0.13^{d}	60.02±0.06^{d}
22:5*n*-3	0.47±0.05^{a}	0.5±0.05^{a}	0.4±0.04^{a}	0.4±0.04^{a}	1.4±0.14^{a}	ND
22:6*n*-3 DHA	4.12±0.4^{a}	7.8±0.78^{a}	7.2±0.73^{a}	6.5±0.66^{a}	28.01±0.8^{b}	29.98±0.03^{b}
∑PUFA	22.25±0.23^{a}	33.96±0.4^{a}	53.3±0.33^{b}	50.09±0.01^{a}	86.69±0.67^{e}	93.5±0.05^{c}
EPA+DHA	16.97±0.77^{a}	30.8±0.08^{b}	44.9±0.49^{c}	43.6±0.36^{a}	79.31±0.93^{d}	90±0.09^{e}
∑*n*-3PUFA	17.7±0.45^{a}	31.51±0.15^{a}	50.4±0.04^{b}	47.2±0.72^{a}	83.41±0.34^{c}	92.01±0.02^{d}
∑*n*-6PUFA	4.5±0.39^{a}	2.4±0.04^{a}	2.88±0.29^{a}	2.86±0.29^{a}	3.26±0.33^{a}	1.48±0.15^{a}
∑*n*-3/∑*n*-6	3.93±0.03^{a}	13.13±0.31^{b}	17.5±0.75^{b}	16.5±0.65^{a}	25.59±0.56^{c}	62.17±0.22^{d}
∑*n*-6/∑*n*-3	0.25±0.94^{a}	0.08±0.01^{a}	0.06±0.01^{a}	0.06±0.01^{a}	0.04±0^{a}	0.02±0^{a}
∑PUFA/∑SFA	0.54±0.05^{a}	1.14±0.11^{a}	2.85±0.29^{a}	2.3±0.03^{a}	33.6±0.36^{b}	41.01±0.1^{c}
∑TRANS	0.11±0.01^{a}	0.12±0.01^{a}	0.04±0^{a}	0.08±0.01^{a}	0.02±0^{a}	0.01±0^{a}

Refined sardine oil – ($E_{6,}$ *Chapter 4*); LHCO - lipase hydrolyzed fatty acid concentrate. LHPO - lipase hydrolyzed purified sardine esters. Data presented as mean values of three samples (means ± standard deviation). These values do not total 100% because minor fatty acids are not reported. ND implies not detected.

5.3. Conclusions

The winterization of PUFA by Me_2O: EtOAc (7:3 v/v) treatment followed by urea complexation and argentation chromatography yielded a final purified PUFA (Total PUFA = 96.7%) rich in *n*-3 fatty acid methyl esters, such as EPA (47.6 %) and DHA (38.2 %). This procedure for concentrating *n*-3 PUFA esters would contribute to the commercial application to obtain concentrated *n*-3 fatty acids, particularly EPA esters from sardine oil. The present study also showed the changes in different classes of fatty acid and composition as a function of time-course (1–6 h) of hydrolysis of triglycerides from sardine oil by a lipase purified from *Bacillus circulans* that may be a potential enzyme source for concentration of *n*-3 PUFAs of acyl chain length shorter than C20. It was found that ester bonds of n-3 fatty acid homologues were highly resistant to hydrolysis by lipase from *B. circulans*, resulting in their enrichment in fatty acid triglycerides. Structure–bioactivity relationship analyses revealed a direct relationship of hydrophobic and steric properties to lipase-catalyzed hydrolytic effects on fatty acyl ester bonds of triglycerides. The fractions eluted with *n*-hexane/Me_2O (95:5, v/v) and *n*-hexane/EtOAc (9:4, v/v), by argentated chromatography furnished 20:5*n*-3 ester in final purities of 68.3 % of total fatty acids. A combination of *B. circulans* lipase-catalyzed hydrolysis followed by urea fractionation and argentated chromatography, is a promising method for separating and concentrating *n*-3 fatty acids such as 20:5*n*-3 from sardine oil.

Chapter 6

PREPARATION OF DIFFERENT NATURAL ADDITIVES TO STABILIZE SARDINE OIL

Contents

Background

Traditional methods to reduce the process of lipid oxidation utilize synthetic phenolic antioxidants such as BHA, BHT, t-BHQ, propyl gallate etc. But, since these synthetic antioxidants generate health risks and toxicity there is an increased interest in the antioxidants of natural origin in recent times. In addition, the use of both synthetic and certain commercially available natural antioxidants such as ascorbic acid and tocopherols have been shown to be inefficient in some foods enriched with marine long chain *n*-3 PUFAs. Due to the high level of LC-PUFA in the marine fish oils, natural antioxidants need to display comparative or better oxidative capacity than readily available synthetic chemicals. Numerous studies have focused on the natural antioxidants in terrestrial plants and their application in marine oils to prevent the oxidation. Hence, the antioxidative potential of various aquatic resources (halophytes and red seaweeds) along with terrestrial sources (rosemary and green tea) were used to study their efficacy to impart antioxidant activity.

Salt marsh halophytic plants are known for their ability to withstand and quench the reactive oxygen species, since they are equipped with a powerful antioxidant system that includes enzymatic and non-enzymatic components (Lee *et al*., 2011). Halophytes or salt tolerant plants are able to grow in saline to extremely saline habitats, and have particular characteristics, which enable them to evade and/or tolerate salinity by various eco-physiological mechanisms. These plants are naturally grown or cultivated in salt-affected lands such as in saline semi-deserts,

mangrove swamps, marshes, sloughs, degraded soils and seashores (Ksouri *et al*., 2008; Megdiche *et al*., 2007).

Seaweeds are photosynthetic organisms, and are highly exposed to a combination of stressful factors, viz., light and oxygen at the origin of the formation of free radicals and other oxidative reagents. However the absence of oxidative damage in the structural components of seaweeds suggests that their cells have protective antioxidative defense systems (Chakraborty & Paulraj 2010; Escrig *et al*., 2001). Therefore, this marine flora may be considered as a potential resource of natural antioxidant molecules. Among various red seaweeds found in the Gulf of Mannar region in the southeastern coast of the Indian subcontinent, *Kappaphycus alvarezii*, G*racilaria corticata*, *Hypnea musciformis*, *Hypnea valentiae* and *Jania rubens* are abundantly available throughout different seasons. Although antioxidant properties of seaweeds were proved by numerous studies from the past two decades, there is scanty information regarding the antioxidant potential of these species from this very important delta region.

The herbs and spices are not just valuable in adding flavor to foods, but their antioxidant activity also helps to preserve foods from oxidative deterioration thereby increasing their shelf life. Several herbal extracts have been developed and commercialized, showing antioxidant capability that parallels the synthetic antioxidants such as BHT. There is at present increasing interest both in the industry and in scientific community for spices and aromatic herbs because of their strong antioxidant and other medicinal properties, which dominate numerous currently used natural and synthetic antioxidants. Phenolic-rich plant extracts from herbs and spices, e.g., rosemary, green tea etc are effective protectors against oxidative degradation in an immense variety of food products.

This chapter focused to understand the antioxidant potential of the halophytes, seaweeds and natural herbal extracts by studying their antioxidant-related properties in a battery of several *in vitro* assays.

6.1. Materials and Methods

6.1.1. Chemicals, Reagents and Instrumentation

All solvents used for the sample preparation were of analytical grade (E-Merck, Darmstadt, Germany). These solvents were redistilled in an all-glass system. The solvents were N_2 degassed prior to use. Doubly distilled water was used throughout this work, while all other reagents used were of analytical grade and purchased from E-Merck (Darmstadt, Germany). Na_2CO_3, gallic acid, BHT (butylated hydroxy toluene), 2,2′-azino-bis-3 ethylbenzothiozoline-6-sulfonic acid diammonium salt (ABTS salt), 1, 1-diphenyl-2-picryl- hydrazil (DPPH•), Folin–Ciocalteu reagent, potassium persulfate ($K_2S_2O_8$), ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), ascorbic acid, ammonium acetate, acetyl acetone, glacial acetic acid, hydrogen peroxide (H_2O_2), thiobarbituric acid (TBA), potassium ferric cyanide, ferric chloride, ferrozine, bovine serum albumin, trichloroacetic acid (TCA), $FeSO_4$ were purchased from Merck or Sigma Aldrich or HiMedia. The standards of epigallocatechin gallate (EGCG, ≥ 99%), (+)-catechin, epicatechin, epicatechin gallate (ECG; ≥ 98% by HPLC) and catechin gallate (CG; ≥ 99% by HPLC), carnosic acid (from *Rosmarinus officinalis* ≥ 91% powder) were purchased from Sigma-Aldrich (Germany). All other unlabeled chemicals and reagents were of analytical, spectroscopic or chromatographic reagent grade and were obtained from E-Merck (Darmstadt, Germany).

A laboratory scale lyophilizer (Alpha 1-4 LD plus, Germany) was used for freeze-drying. The solvents were evaporated in a rotary vacuum evaporator (Heidolf, Germany). The absorbance was measured in an UV–VIS spectrophotometer (Varian Cary, USA), and an ultra-centrifuge (Sorvall, Biofuge Stratos, Thermo Scientific, Germany) was used for centrifugation. A HPLC system (Shimadzu, LC-20AD) was used for analyzing the presence of phenolic compounds in the rosemary and green tea extracts.

6.1.2. Samples and Study Area

6.1.2.1. Halophytes

The five halophyte plants selected in this study were,

- *Salicornia brachiata* Miq.,
- *Arthrocnemum indicum* Nels.,
- *Suaeda maritima* L. Dumort.,
- *Suaeda monoica* Forssk ex JF Gmel.

} Family - Chenopodiaceae

- *Sesuvium portulacastrum* L., (Family Azoaceae)

These halophytes were collected from the Southeast coast of the Indian subcontinent from Punnakayal (situated about 26 km Southeast of Tuticorin) in the Gulf of Mannar region on the Southeast coast of India (8º48' N 78º11' E) (Fig. 6.1). The photographs of the halophytic plants, (B1) *Salicornia brachiata*; (B2) *Suaeda maritima*; (B3) *Arthrocnemum indicum* and (B4) *Sesuvium portulacastrum* are shown in Fig. 6.2.

Fig. 6.1. Sampling site of the halophytes showing Punnakayal of Tuticorin district in Southeast coast of India

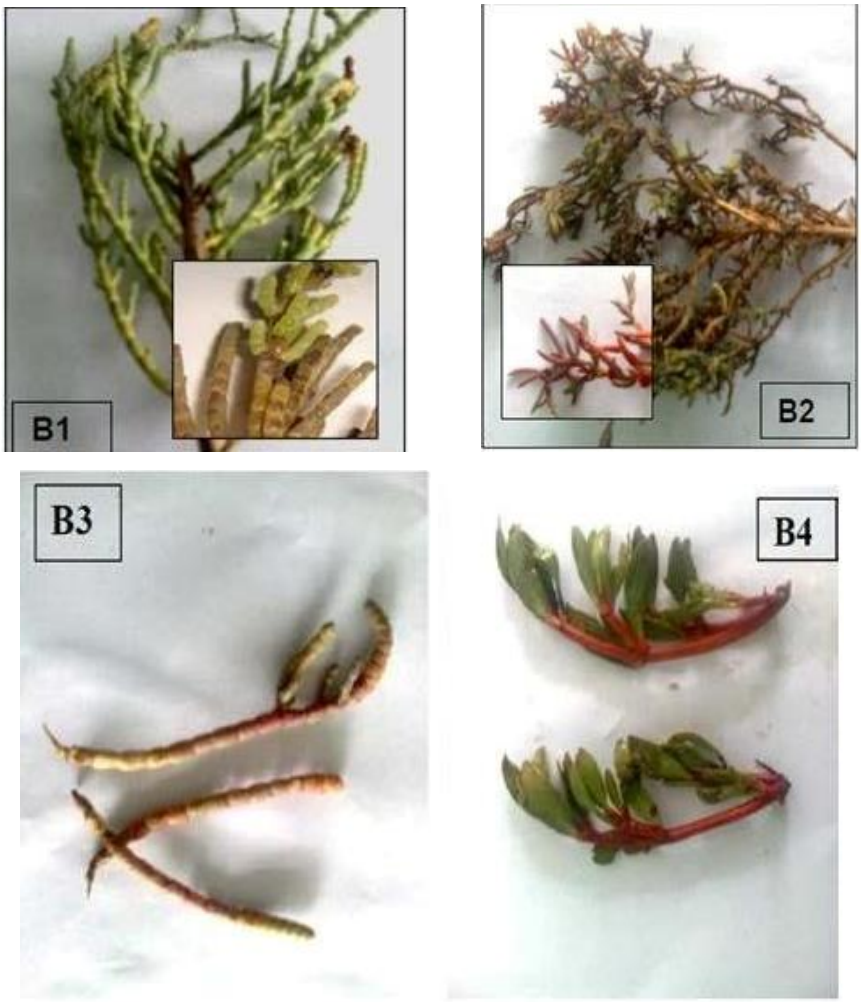


Fig. 6.2. Photographs of the halophytes, (**B1**) *Salicornia brachiata;* (**B2**) *Suaeda maritima;* (**B3**) *Arthrocnemum indicum* and (**B4**) *Sesuvium portulacastrum*

6.1.2.2 Seaweeds

Five red seaweeds studied were as follows,

- *Kappaphycus alvarezii (Doty) Doty ex P.C.Silva* [Family - Solieriaceae; Order - Gigartinales]
- *Gracilaria corticata (J.Agardh) J.Agardh 1852* [Family - Gracilariaceae; Order - Gracilariales]
- *Hypnea musciformis* (Wulfen) J.V. Lamouroux [Family - Hypneaceae; Order -Gigartinales]

- *Hypnea valentiae* (Turner) Montagne [Family - Hypneaceae; Order - Gigartinales]
- *Jania rubens* (Linnaeus) J.V. Lamouroux [Family - Corallinaceae; Order - Corallinales]

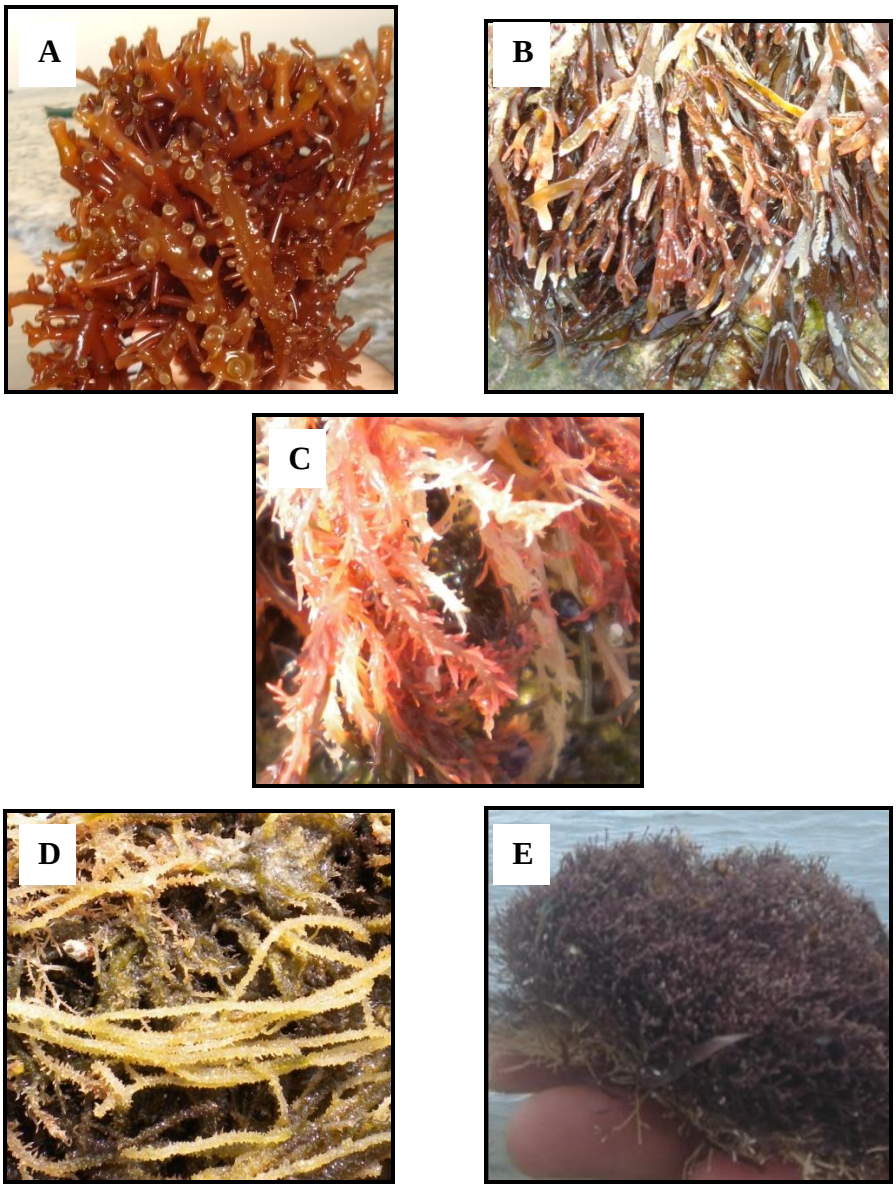


Fig. 6.3. Photographs of (**A**) *K. alvarezii*; (**B**) *G. corticata*; (**C**) *H. musciformis*; (**D**) *H. valentiae* and (**E**) *Jania rubens* collected from the Gulf of Mannar region in Southeast coast of India

These seaweeds were freshly collected from the Gulf of Mannar in Mandapam region located between 8º48′ N, 78º9′ E and 9º14′ N, 79º14′E on the Southeast coast of India (Fig. 6.4).

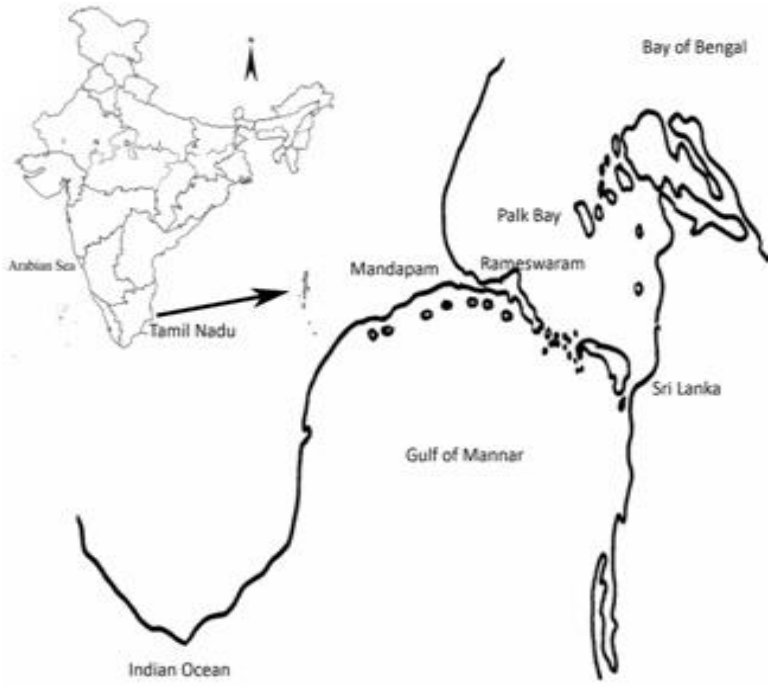


Fig. 6.4. Sampling area of the red seaweeds showing Gulf of Mannar region in Southeast coast of India (Lat 8°48′ N; Long 78°9′ E and Lat 9°14′ N; Long 79°14′E).

6.1.2.3. Herbs

The leaves of rosemary (*Rosmarinus officinalis* L.) and green tea (*Camellia sinensis* O. Kuntze) were purchased from the local market in Cochin, Kerala (Fig. 6.5). Rosemary (*Rosmarinus officinalis*), is a woody, perennial herb with fragrant, evergreen, needle-like leaves and white, pink, purple or blue flowers, native to Mediterranean and Asia regions. It is a member of the mint family (Lamiaceae).

Fig. 6.5. Photographs of (**A**) *Rosmarinus officinalis* and (**B**) *Camellia sinensis*

6.1.3. Preparation of Extracts from Natural Additives

6.1.3.1. Marine Halophytes

One kilogram of each halophyte was cleaned with running water to remove the extraneous materials, ground in a mixer grinder and lyophilized (24 h) in a laboratory lyophilizer to obtain the lyophilized halophyte samples (800 g each). About 100 g each of the lyophilized halophytes was extracted with EtOAc (1 l X 3), filtered through Whatman No.1 filter paper, and evaporated (50°C) *in vacuo* (Heidolph, Germany) to furnish EtOAc extracts of halophytes. The extracts were stored at 4°C until further analysis.

6.1.3.2. Marine Seaweeds

The powdered seaweeds (100 g) were extracted three times with MeOH (50–60 °C, 3 h), filtered through Whatman No. 1 filter paper, and the pooled filtrate was concentrated (50 °C) *in vacuo* to one-third volume, and then partitioned successively with *n*-hexane (150 ml ×3), DCM (150 ml ×3) and EtOAc (150 ml ×3), concentrated *in vacuo* to furnish *n*-hexane, DCM, and EtOAc fractions, respectively.

6.1.3.3. Natural Herbal Extracts

The fresh rosemary leaves (50 g) were air dried, ground, and were subjected to soxhlet extraction (75-80^{0}C) using EtOAc. The pooled extract was filtered and concentrated to obtain EtOAc extract (5.6 g). The extract was then stirred with *n*-hexane (25 ml; 20 min), centrifuged and the supernatant was concentrated to obtain the refined extract of *R. officinalis* (2.1 g).

The ground green tea, *Camellia sinensis*, leaves (10 g) were stirred with 50% aqueous EtOH (100 ml) at room temperature and filtered. The filtrate was mixed with an equal volume of chloroform, separated and the upper aqueous layer was extracted three times with an equal volume of EtOAc. The EtOAc extracts were combined and concentrated to obtain the refined extract of *Camellia sinensis* (2.8 g).

The HPLC analysis of the rosemary and green tea were performed in order to confirm the presence of phenolic compounds in their extracts. The HPLC was performed using a reverse phase HPLC system (Shimadzu LC-20AD) fitted with a

C_{18} column (Phenomenex, 250 mm length, 4.6 mm i.d, 5µ particle size) and photo diode array (PDA) detector. Carnosic acid and carnosol were detected at 230 nm by eluting with acetonitrile: 1% acetic acid (55:45, v/v; isocratic) and green tea polyphenols were detected at 280 nm by using (A) 0.05% H_3PO_4 (pH 2.4) and (B) CH_3OH:CH_3CN (3:2) in a binary gradient programme as follows: 0 -15 min (10% - 25% B), 15.1-25 min (25% - 60% B), 25.1-40 min (60% - 10% B),

The extracts of both rosemary and green tea were mixed in various ratios *viz*., 80:20; 60:40; 50:50, 40:60 and 20:80, respectively (%, w/w) and studied for different *in vitro* antioxidant activity assays.

6.1.4. Antioxidant Activity Assays

6.1.4.1. Determination of Total Phenolic Compounds

Total phenolic content in the halophyte EtOAc extracts, seaweed MeOH extract/fractions, herbal extracts/combinations were determined according to the method as described earlier (Lim *et al*., 2007) with minor modifications. Briefly, 0.5 ml of the extracts/fractions (5 mg/ml in MeOH) was added into a test tube containing 2.25 ml MeOH. After the addition of 0.25 ml of Folin–Ciocalteu reagent, the mixtures were stirred for 1 min and allowed to stand for 8 min. About 2.0 ml of sodium carbonate (7.5%, w/v) was added and the mixtures were incubated for 120 min at 25 ^{0}C. The absorbance, relative to that of the blank (MeOH), was measured at 756 nm using a UV–VIS spectrophotometer. A standard curve was plotted using different concentrations of gallic acid and the amount of total phenolics was calculated as milligram of gallic acid equivalents (mg GAE)/g of the extracts or fractions. All determinations were performed in triplicate.

6.1.4.2. Free Radical Scavenging Ability Assays

6.1.4.2A. 1, 1-Diphenyl-2-Picryl- Hydrazil (DPPH•) Radical Scavenging Activity

The antioxidant activities of the halophyte EtOAc extracts, seaweed MeOH extract/fractions and the herbal extracts/combinations was measured using the stable radical, $DPPH^{\cdot}$, as a standard reagent. This was determined as described earlier (Lim *et al*., 2007) with suitable modifications. Briefly, the stock solutions of the

extracts/fractions were prepared in MeOH. Dilutions were made to obtain concentration ranging from 0.1 to 1.0 mg/ml. Diluted solutions (2.0 ml) were mixed with 2.0 ml of 0.16 mM $DPPH^{\cdot}$ in MeOH. The mixtures were shaken vigorously and maintained for 30 min at ambient temperature (30 ^{0}C) in the dark. The absorbance of mixtures was measured at 517 nm against a reagent blank by using a UV–VIS spectrophotometer. The absorbance of the control (2 ml $DPPH^{\cdot}$ solution + 2 ml MeOH) and the samples were measured spectrophotometrically at different time intervals (0, 0.5, 1.0, 4.0, 8.0, 16, 24, 48, 72, 96 h). The tests were performed in triplicate. The scavenging activity can be calculated by using the following equation: $DPPH^{\cdot}$ scavenging activity (%) = (A_{517} of control − A_{517} of sample) X 100/A_{517} of control. The plot of scavenging activity on $DPPH^{\cdot}$ was recorded and the IC_{50} value (concentration of the sample to scavenge 50% of the DPPH radicals; mg/ml) was then calculated. IC_{50} values (mg/ml) were determined from the linear regression curve of antioxidant activities against the different concentrations of seaweed extracts/ fractions.

6.1.4.2B. 2, 2'-Azino-bis-3 Ethylbenzothiozoline-6-Sulfonic acid Diammonium salt (ABTS) Radical Scavenging Activity

The free radical scavenging activity of the seaweed MeOH extract/fractions was also determined by $ABTS^{\cdot+}$ ion decolourization assay as described earlier (Vijayabaskar & Shiyamala 2012) with suitable modifications. Briefly, $ABTS^{\cdot+}$ was dissolved in deionized water to 7 mM concentration, and potassium persulfate ($K_2S_2O_8$) is added to a concentration of 2.45 mM. The reaction mixture was left to stand at room temperature overnight (12-16 h) in the dark before use. The resultant intensely-colored $ABTS^{\cdot+}$ was diluted with MeOH to give an absorbance of ~0.70 at 734 nm. The $ABTS^{\cdot+}$ scavenging activity was assessed by mixing 5ml of the above $ABTS^{\cdot+}$ solution with 0.1 ml extracts/fractions (0.1, 0.2, 0.3, 0.4 and 0.6 µg/ml). The final absorbance was measured at 743 nm with UV – VIS spectrophotometer. The percentage of scavenging was calculated by the following formula: $ABTS^{\cdot+}$ scavenging activity (%) = ((A_0-A_1) / A_o) x 100, where A_0 is the absorbance of control

and A_1 is the absorbance of the sample. The plot of scavenging activity on $ABTS^{\cdot+}$ radical was recorded and the IC_{50} value (µg/ml) was then calculated.

6.1.4.3. Hydroxyl ($HO^{\cdot}$) Radical Scavenging Activity

The capacity of the seaweed MeOH extract/fractions to scavenge hydroxyl radicals was measured according to a modification method described by Singh *et al*. (2002). The extracts/ fractions in different concentrations (0.1, 0.2, 0.4 and 0.6 mg/ml) in MeOH were evaporated in vials and the reaction mixture contained 1 ml of iron-EDTA solution, 0.5 ml of EDTA (0.018%), 1 ml of DMSO (0.85% v/v in 0.1M phosphate buffer, pH 7.4) and 0.5 ml of ascorbic acid (0.22%) was added to each tube. The vials were capped tightly and heated in a water bath at 80-90 °C for 15 min. The reaction was terminated by adding 1 ml of ice-cold TCA (17.5%). Three ml of Nash reagent (75.0 g of ammonium acetate, 3 ml of glacial acetic acid and 2 ml of acetyl acetone and adjust the volume up to 1L) was added to each vial and left at room temperature for 15 min for color development. The intensity of yellow color was measured spectrophotometrically at 412 nm against blank sample. The mixture without sample was treated as control. All tests were performed in triplicate. The scavenging activity was calculated by following equation: % hydroxyl radical scavenging activity = $[(A_0-A_1) / A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the samples. The plot of $HO^{\cdot}$ radical scavenging activity by different concentrations was recorded and the IC_{50} value (mg/ml) was then calculated.

6.1.4.4. Hydrogen Peroxide (H_2O_2) Scavenging Activity

The ability of the seaweed extracts/fractions to scavenge H_2O_2 was determined according to the method of Ruch *et al*. (1989) with slight modifications. Briefly, 40 mM H_2O_2 was prepared in phosphate buffer (pH 7.4). The extracts/fractions of different concentrations (0.1 - 1.0 mg/ml) in MeOH (3.0 ml) were added to 3.0 ml of 40 mM H_2O_2 solution and the absorbance of H_2O_2 was determined at 230 nm after 10 min incubation against a blank solution containing phosphate buffer without H_2O_2. The percentage scavenging of H_2O_2 was calculated as: % H_2O_2 scavenging activity = $[(A_0-A_1) / A_0] \times 100$, where A_0 is the absorbance of

control and A_1 is the absorbance of sample. The plot of scavenging activity on H_2O_2 was recorded and the IC_{50} value (mg/ml) was then calculated.

6.1.4.5. Lipid Peroxidation Inhibition Activity in Model System: Thiobarbituric Acid-Reactive Species (TBARS) Formation Inhibitory Activity

The TBARS formation inhibitory assay of halophyte EtOAc extracts, seaweed MeOH extract/fractions and herbal extracts/combinations was performed by earlier described method (Chakraborty *et al*., 2013) with suitable modifications. The model system used for this assay was lyophilized green mussel (*Perna viridis* L.) as a lipid source {*Green mussels were collected from their natural habitat, Elathur (Lat: 11° 20′ 0″ N, Long: 75° 44′ 0″ E) in Kerala (Southwest coast of India). The meat taken out was freeze-dried a laboratory freeze-drier*}. The lyophilized powder of *P. viridis* meat (10 mg) was incubated with 1 ml of different seaweed extracts/fractions (1 ml; 2 mg/ml). The incubation was stopped by the addition of cold acetic acid (2 ml, 20 % v/v; pH 3.6), and the malondialdehyde (MDA) formation was followed by the addition of TBA (2 ml, 0.78 % w/v in acetic acid). The mixtures were incubated at 95 ^{0}C for 45 minutes, cooled to room temperature and centrifuged (8000 rpm, 10 min). The absorbance was measured at 532 nm. TBARS formation inhibitory activity was expressed as mM of malondialdehyde equivalent compounds (MDAEQ) formed per kilogram of the sample (MDAEQ/kg) related to the control (lyophilized green mussel) undergoing maximum lipid peroxidation on the same assay conditions.

6.1.4.6. Reducing Ability

The reducing power/ability of the seaweed MeOH extract/fractions was measured according to the method of (Lim *et al*., 2007) with some modifications. 1.0 ml of extracts/fractions (1 mg/ml in MeOH) was mixed with 2.5 ml phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferric cyanide. The mixture was incubated at 50 ^{0}C for 20 min. After incubation, 2.5 ml of 10% TCA was added to the mixture and centrifuged at 6000 g for 10 min. Then, 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. The reducing ability was represented as the absorbance measured at 700 nm ($Abs_{700\ nm}$)

after 10 min. Increased absorbance of the reaction mixture indicated increased reducing power. All the measurements were in triplicates.

6.1.4.7. Ferrous ion (Fe^{2+}) Chelating Activity

The ferrous ion chelating activity of the halophyte EtOAc extracts, seaweed MeOH extract/fractions, herbal extracts/combinations was measured as described earlier (Lim *et al*., 2007) with suitable modifications. One ml 0.125 mM $FeSO_4$, and 1.0 ml 0.3125 mM ferrozine were mixed with 1.0 ml extracts/fractions (0.1, 0.2, 0.4 and 0.6 mg/ml). The mixture was allowed to equilibrate for 10 min before measuring the absorbance. The decrease in the absorbance at 562 nm of the iron (II)-ferrozine complex was measured. The ability of the extracts/fractions to chelate Fe^{2+} was calculated relative to the control (consisting of Fe and ferrozine only) using the equation: % chelating ability = $(A_0 - A_1) \times 100/A_0$, where A_0 is the absorbance of control and A_1 is the absorbance of sample. The concentration of extracts/fractions required to get 50% iron chelating activity, called effective concentration (IC_{50}) was also calculated.

6.1.5. Statistical Analysis

Differences between means at the 5% ($p<0.05$) level were considered significant. The significant differences ($p < 0.05$) among the samples were identified using Statistical Program for Social Sciences 13.0 (SPSS, USA, ver. 13.0) through ANOVA and Duncan analyses. To evaluate the relationship between different antioxidant activities and polyphenolic contents (TPC), Pearson correlation analysis were performed.

6.2. Results and Discussion

6.2.1. Halophytes

6.2.1.1. Yield

The percent yield (% w/w of lyophilized halophytes) of the EtOAc extracts of *S. brachiata* and *S. portulacastrum* recorded maximum yield (0.5 and 0.6 g/100g, respectively) as compared with *S. maritima* (0.36 g/100g), *A. indicum* (0.34 g/100g) and *S. monoica* (0.31 g/100g).

6.2.1.2. Antioxidant Activities of Halophyte Extracts

The antioxidant activities of the EtOAc extracts of halophytes were determined by the total phenolic content (TPC), DPPH radical scavenging activity, TBARS formation inhibitory ability and Fe^{2+} ion chelating activity, and the results are discussed as follows.

6.2.1.2A. Total Phenolic Content

The EtOAc extracts of *S. brachiata* and *S. maritima* recorded significant difference (557 and 491 mg GAE/g, respectively) in their total phenolic contents (Fig. 6.6) ($p<0.05$). EtOAc extracts of other halophytes followed the order: *S. monoica* > *S. portulacastrum* > *A. indicum*. An earlier study reported the phenolic content of *S. portulacastrum* as 55.1 mg GAE/g in its aqueous extracts (Agoramoorthy *et al*., 2008). Earlier studies revealed that halophytic algal species possess polyphloroglucinol phenolics (phlorotannins) as phenolic compounds (Nakamura *et al*., 1996). The higher total phenolic contents in these halophytes may possibly be related to the presence of amino acids viz., aspartate, proline, lysine, and glycine, which synthesize numerous substances including phenolics by involving themselves in protein or energy metabolism, and /or transmethylation reactions. The quantity of phenolic compounds were reported to vary greatly among different halophytic Mesembryanthemum spp., with *M. edule* had the highest total phenolic contents (70 mg GAE /g DW), whereas *M. nodiflorum* and *M. crystallinum* exhibited no significant differences in their phenolic contents (Falleh *et al*., 2013).

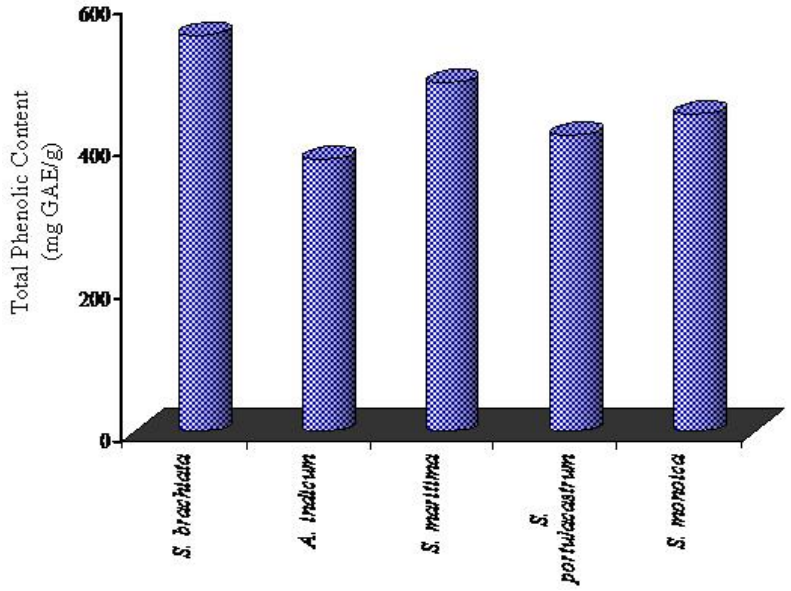


Fig. 6.6. Total phenolic content (mg GAE/g) of the different halophyte extracts

6.2.1.2B. DPPH· Radical Scavenging Activity

The DPPH radical scavenging activities of EtOAc extracts of different halophytes was found to be in the order *S. maritima* (62.7 %) > *S. brachiata* (61.3 %) > *A. indicum* (58.2 %) > *S. monoica* (56.4 %) > *S. portulacastrum* (54.0 %) The IC_{50} values of DPPH radical scavenging activity (IC_{50} 0.89 - 0.96 mg/ml) (Fig. 6.7A) showed that the extracts of these halophytes, especially, *S. maritima* and *S. brachiata* appeared to be the reservoir of compounds responsible to scavenge free radicals, and seem to play an important role in their anti-oxidative capacity. In addition, the organic polar solvent extracts (EtOAc) of halophytes may contain polyphenolic compounds capable of deactivating DPPH free radical. These observations clearly depict that the antioxidant properties exhibited by potential halophytic species in the present study may be due to the presence of phlorotannins and polyphenolic compounds, or any other potential antioxidants present with them. It can be generalized that EtOAc extract of *S. brachiata* and *S. maritima* possess reasonably good polyphenolic content with free radical scavenging activity, and are the candidate halophytic species to further explore the compounds responsible for radical scavenging activities. DPPH scavenging activity was found to be positively correlated with total phenolic content (r^2 = 0.448) (Fig. 6.7B) thereby realizes the

role of phenolic compounds responsible for the antioxidant properties of halophyte extracts.

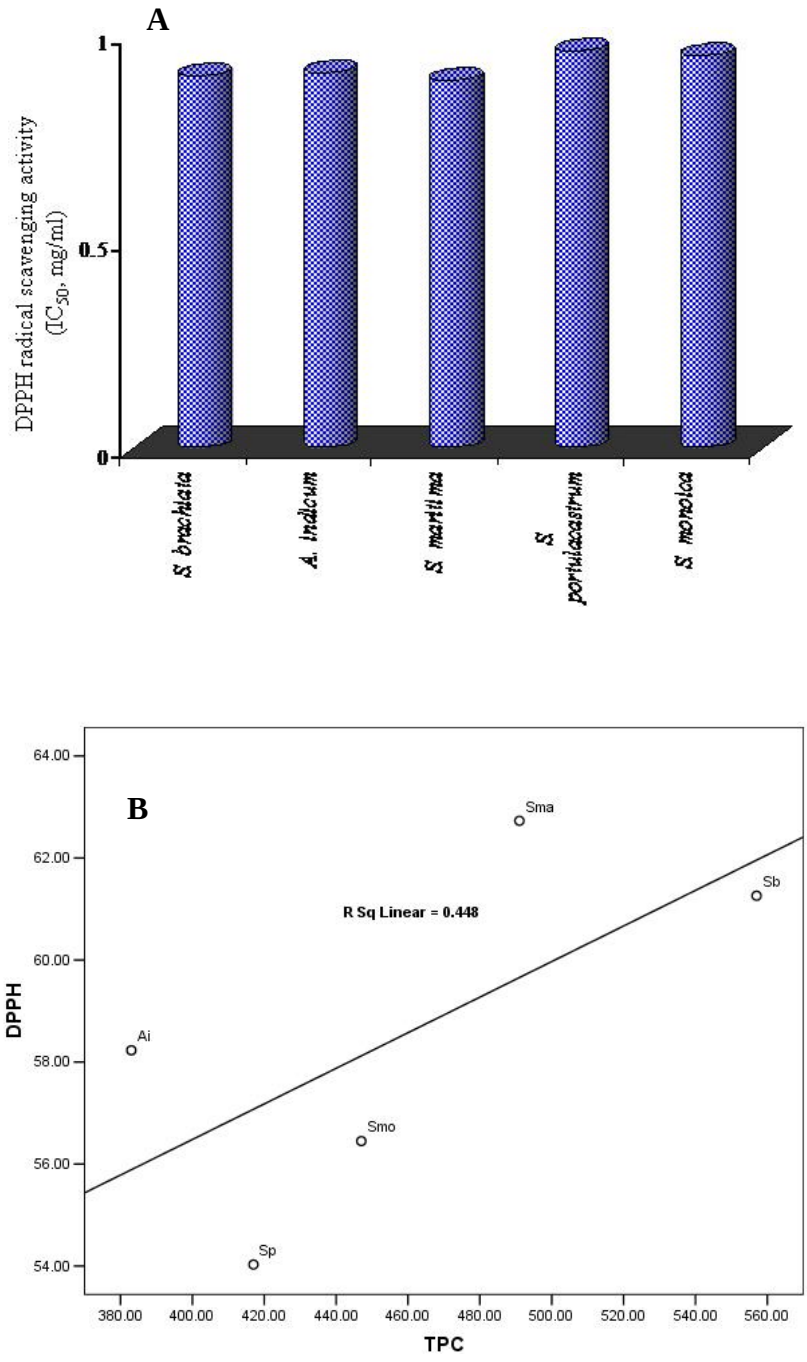
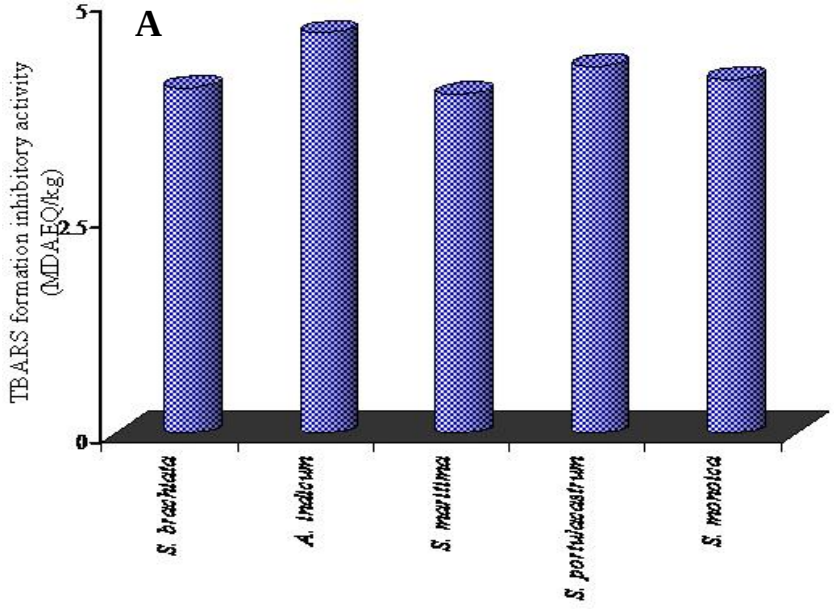


Fig. 6.7. (**A**) DPPH· scavenging activity (IC_{50}, mg/ml) and (**B**) Scatter plot diagram showing the correlation of total phenolic content & DPPH· scavenging activity of different halophyte extracts

6.2.1.2C. Thiobarbituric Acid-Reactive Species (TBARS) Formation Inhibitory Activity

TBARS assay, which reflect the production of low molecular weight end products, like malondialdehyde is used to indicate free-radical generation. TBARS formation inhibitory activity at 2 mg/ml of the EtOAc extracts of the five halophytes was recorded in Fig. 6.8A. *S. maritima* (3.94 MDAEQ/kg) and *S. brachiata* (4.01 MDAEQ/kg) extracts exhibited maximum TBARS formation inhibitory ability than the other studied halophytes. The inhibition of lipid peroxidation may be due to the presence of polyphenolic antioxidants that were reported to disrupt free-radical chain reaction by donating a proton to fatty acid radicals to terminate chain reactions (Karawita *et al*., 2005). However, a negative correlation realized between TPC and lipid peroxidation inhibition activity ($r^2 = 0.673$) (Fig. 6.8B), obviously indicated that antioxidant activity did not depend only on TPC, but also on other factors or there may be some active metabolites other than phenolics such as polysaccharides capable of inhibiting the TBA-MDA adduct formation (Chakraborty *et al*., 2013).



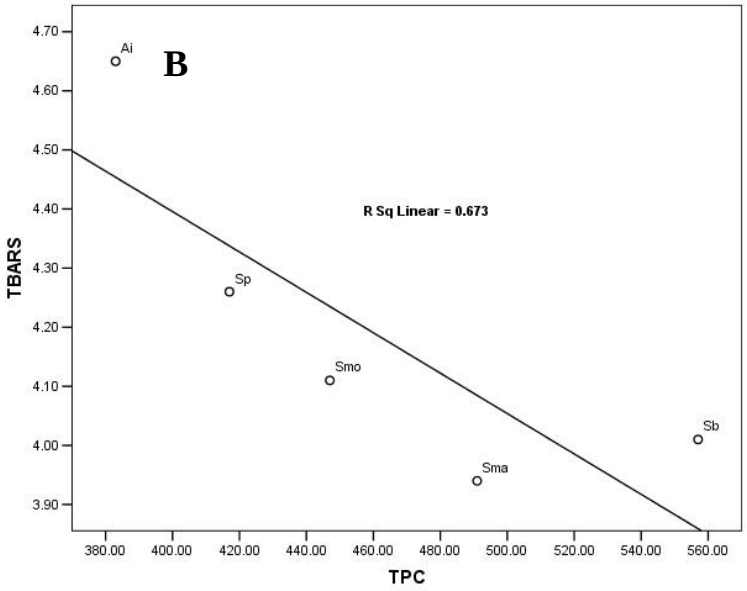


Fig. 6.8. (**A**) TBARS formation inhibitory activity (MDAEQ/kg) and (**B**) Scatter plot diagram showing the correlation of total phenolic content & TBARS formation inhibitory activity of different halophyte extracts

6.2.1.2D. Ferrous ion Chelating Activity

Fe^{2+} ion chelating activity (%) at 0.6 mg/ml of the halophyte extracts (Fig. 6.9A) were in the order: *S. brachiata* > *S. maritima* > *S. monoica* > *S. portulacastrum* > *A. indicum*. This result clearly indicated the secondary antioxidant mechanism property of *S. brachiata* and *S. maritima* that inhibits the prooxidant transition metals from catalyzing the breakdown of hydroperoxides. A high positive correlation (r^2 = 0.86) (Fig. 6.9B) between Fe^{2+} ion chelating activity and TPC shows the ability of the phenolic compounds present in the halophyte extracts to compete with ferrozine in chelating ferrous ion. The OH groups in the phenolic compounds could be able to chelate certain metal ions. Andjelković *et al*. (2006) observed that phenolic acids which have catechol or galloyl moiety in their structure were able to show iron chelating property.

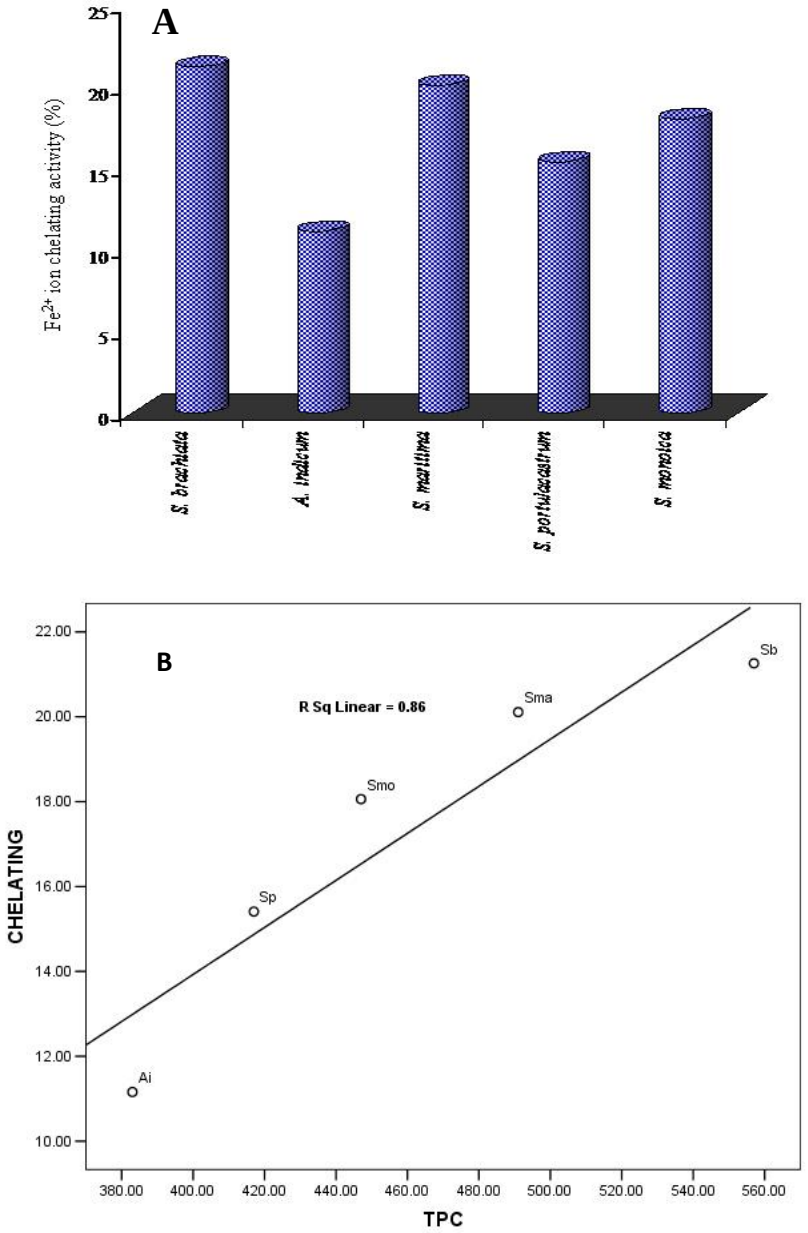


Fig. 6.9. **(A)** Fe^{2+} ion chelating activity (%) and (**B**) Scatter plot diagram showing the correlation of Total phenolic content & Fe^{2+} ion chelating activity of different halophyte extracts

6.2.2. Red Seaweeds

6.2.2.1. Yield

The yields of the MeOH extracts, *n*-hexane, DCM, and EtOAc fractions of *H. musciformis* , *H. valentiae* and *J. rubens* were shown in Fig. 6.10. Among the MeOH extracts of five seaweeds, *H. valentiae* exhibited higher yield (6.5 g/ 100 g dry sample) followed by *J. rubens*, *H. musciformis*, *G. corticata* and *K. alvarezii* (5.3, 4.8, 3.0 & 1.3 g/100 g dry sample, respectively). The yield of methanolic extract in the present study was higher as compared to the earlier study by Nguyen and Kim (2012) who obtained 4.6 g/100 g dry sample of total MeOH extract in the red seaweed, *Grateloupia lancifolia*. Among the different solvent fractions, *n*-hexane fraction had the highest yield in all the seaweeds (6.3 – 30.6 g/100 g MeOH extract). The higher yield of *n*-hexane fractions compared to the DCM and EtOAc fractions of the same species showed that most of the compounds in these seaweeds were low in polarity and lipophilic.

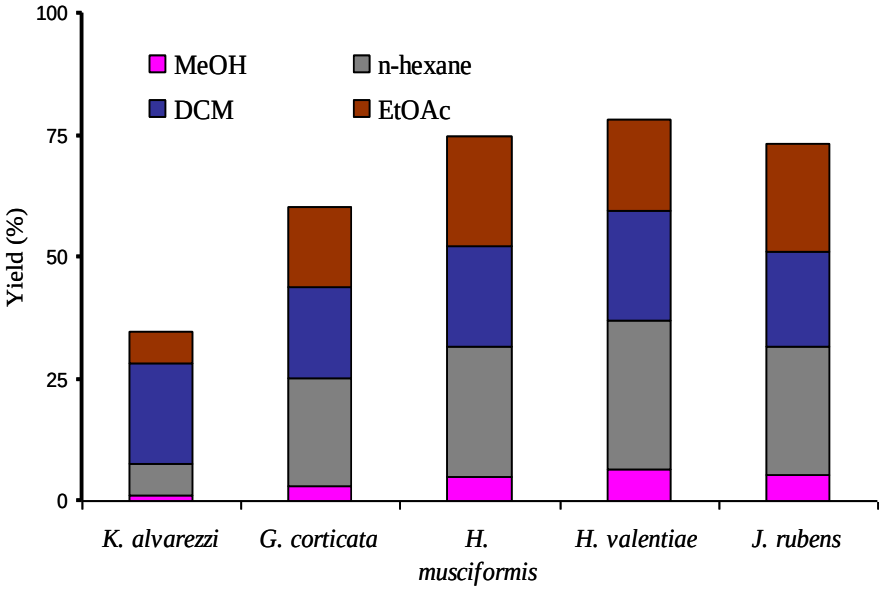


Fig. 6.10. Percent yield (%, w/w) of the MeOH extract and three solvent fractions (*n*-hexane DCM and EtOAC) of the red seaweeds

6.2.2.2. Antioxidant Activity of Red Seaweeds

The antioxidant activities of the MeOH extracts and different solvent fractions of the red seaweeds were determined by the total phenolic content (TPC), DPPH radical scavenging activity, 2,2′-azino-bis-3ethylbenzothiozoline-6-sulfonic acid diammonium salt (ABTS) scavenging activity, hydroxyl radical scavenging activity, scavenging of hydrogen peroxide, TBARS formation inhibitory ability and Fe^{2+} ion chelating activity assays and the results are as follows.

6.2.2.2.1. Total Phenolic Content of Red Seaweeds

The total phenolic contents observed for extracts/fractions (5mg/ml) are shown in Table 6.1. MeOH extract of *H. musciformis* and *H. valentia* exhibited significantly higher phenolic content (9.8 & 6.9 mg GAE/g, respectively) ($p<0.05$) compared with *J. rubens*, *G. corticata* and *K. alvarezii* (4.9, 3.4 & 1.7 mg GAE/g, respectively). EtOAc fraction of the seaweeds exhibited high phenolic content (37 - 205 mg GAE/g) followed by DCM (1.3 - 87 mg GAE/g) and *n*-hexane (2 - 57 mg GAE/g) fractions. These results corroborated well with those studies by Duan *et al*. (2006), who observed high phenolic content in the EtOAc soluble fraction of red seaweed, *P. urceolata* (73.7 GE/g). In the present study, it was noticed that for all the five seaweeds, higher contents of the phenolic compounds were observed in the solvent fractions than the crude MeOH extract. Similar finding was also reported by Ganesan *et al*. (2008). This could be due to more interfering substances present in the crude extract as compared to different solvent fractions. Moreover, total phenolic content increased in the fractions with increasing solvent polarity (eg. EtOAc and DCM). It is evident from the present observations that a significantly higher ($p<0.05$) phenolic content was observed in the polar solvent fractions (EtOAc fraction) of *Hypnea* sp indicating their high antioxidant potential (Table 6.1). Zubia *et al*. (2007) reported that another *Hypnea* sp, *Hypnea spinella* collected from the Gulf of Mexico and Caribbean coast of Yucatan and Quintana Roo showed a phenolic content of 0.67 % (dw). Interestingly, the phenolic content in the EtOAc fraction of *H. musciformis* was significantly higher (205.5 mg GAE/g) compared to *H. valentiae* (72.9 mg GAE/g), *K. alvarezii* (54.3 mg GAE/g), *J. rubens* (37.3 mg GAE/g) and *G. corticata* (34.2 mg GAE/g) (Table 6.1). There are earlier reports that

substantiate our present observation that solvent extracts of *Hypnea* sp and other red seaweeds are good resources of polyphenolic compounds (Pavia & Aberg 1996; Yoshie *et al*., 2000).

6.2.2.2.2. Free radical Scavenging Activity of Red Seaweeds

6.2.2.2.2A. 1, 1-Diphenyl-2-picryl-hydrazil (DPPH•) Scavenging Activity

DPPH radical scavenging activities (%) of the extracts/ fractions were found to be maximum at the 48th h. The scavenging activity observed at 1 mg/ml (48th h) is shown in Table 6.1. MeOH extract of *J. rubens* and *H. musciformis* and showed significantly higher ($p<0.05$) DPPH· scavenging activities (17.7 & 15.4 %, respectively) than *K. alvarezii* (11 %), *H. valentiae* (7.7 %) and *G. corticata* (6.5 %). However, the EtOAc fraction of *H. musciformis* registered significantly higher DPPH· scavenging activity (83 %) than *K. alvarezii*, *J. rubens*, *H. valentiae* and *G. corticata* (61.3, 29.9, 20.5 & 18.4 %, respectively). Earlier studies showed high DPPH radical scavenging activities in the EtOAc fractions of red seaweeds, *Rhodomela confervoides*, *Polysiphonia urceolata* and *Ecklonia cava* (Duan *et al*., 2006; Wang *et al*., 2009; Senevirathne *et al*., 2006), methanolic and aqueous extracts from red seaweeds, *Gracilaria verrucosa*, *G. textorii*, *Grateloupia filicina*, *Polysiphonia japonica*, *Euchema Kappaphycus* and *Gracilaria edulis* (Heo *et al*., 2006; Ganesan *et al*., 2008). Apparently, in *H. valentiae* and *J. rubens*, DCM fractions showed significantly higher ($p<0.05$) DPPH·scavenging activity (40.4 & 66.4 %, respectively) than *n*-hexane and EtOAc fractions. According to the IC_{50} values showed in Fig. 6.11A, the DPPH· scavenging ability of the five seaweeds exhibited the following order: *H. musciformis* EtOAc fraction (0.6) > *H. valentiae* DCM fraction (0.75) > *J. rubens* EtOAc fraction (0.94) > *J. rubens* DCM fraction (1.24). The present study suggests that the solvent fractions, especially, EtOAc fractions of *Hypnea musciformis* and *J. rubens* may contain compounds having polyphenolic group/s with multiple -OH groups and/or center of unsaturation in their structural moieties to enable them to donate a proton to DPPH radical thereby neutralizing the latter (Ruberto *et al*., 2001). DPPH· scavenging activity was found to be positively correlated with each other as well as TPC ($r^2 = 0.625$) (Fig. 6.11B)

thereby realizing the role of phenolic compounds responsible for the antioxidant properties of the seaweed extracts.

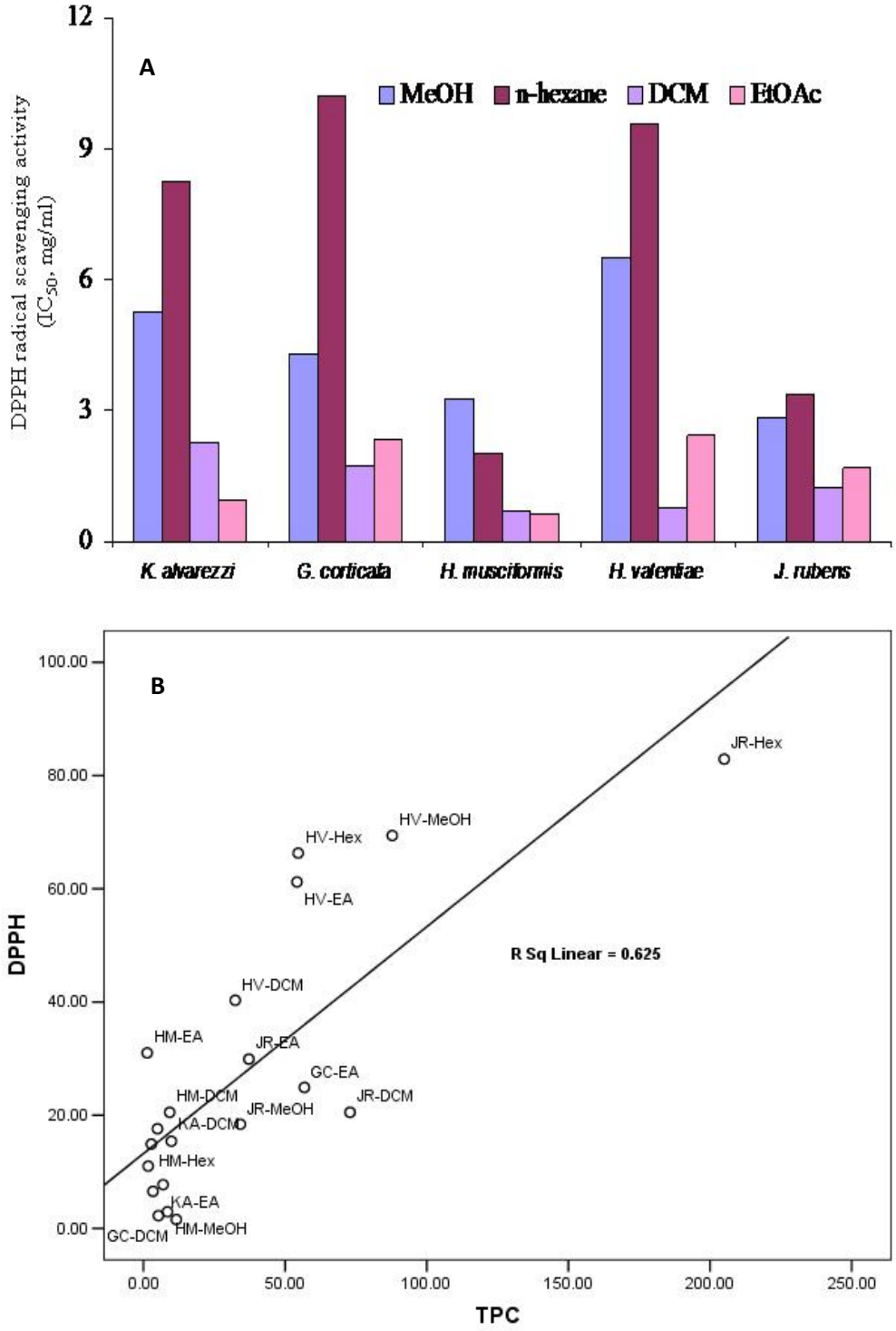


Fig. 6.11. **(A)**IC_{50} values for DPPH scavenging activities (mg/ml) and (**B**) Scatter plot diagram showing the correlation of Total phenolic content & DPPH· scavenging activity of different seaweeds

6.2.2.2.2B. 2, 2'-Azino-bis-3ethylbenzothiozoline-6-Sulfonic Acid Diammonium Salt (ABTS) Scavenging Activity

The ABTS assay has been widely used to investigate free radical scavenging activity of different extracts and fractions and/or its pure compounds. $ABTS^{\cdot+}$ scavenging activities of the MeOH extracts/fractions of the red seaweeds at 0.6 µg/ml are recorded in Table 6.1. Among MeOH extracts, *H. musciformis* recorded significantly higher ABTS.+ scavenging activity (19.6 %) followed by *H. valentiae* (15.0 %), *K. alvarezii* (9.6 %), *J. rubens* (8.7 %) and *G. corticata* (8.0 %). The present observation correlates well with earlier studies, which reported similar antioxidant activities of the MeOH extract of red seaweed, *Padina* sp (Matanjun *et al*., 2008). The EtOAc fraction of *H. musciformis* registered significantly higher $ABTS^{\cdot+}$ scavenging activity (63.3 %) followed by *K. alvarezii* (39.3 %), *H. valentiae* (27.9 %), *G. corticata* (22.2 %) and *J. rubens* (11.1 %) ($p < 0.05$). Interestingly, DCM fraction of *J. rubens* showed significantly higher ABTS.+ scavenging activity (28.9 %) than its MeOH extract and other solvent fractions ($p < 0.05$). The high ABTS.+ scavenging activity realized by EtOAc and DCM fractions of *H. musciformis*, *K. alvarezii* and *J. rubens* could be due to the presence of carotenes/other pigments with long hydrocarbon chain and aminated compounds (Chew *et al*., 2008). Apparently, the mechanism of antioxidant action of this EtOAc and DCM fractions can explain as its H-donating property, thereby terminating the oxidation process by converting free radicals to the stable forms. According to IC_{50} values showed in Fig. 6.12A, the $ABTS^{\cdot+}$ scavenging ability of the extracts/fractions of five seaweeds exhibited the following order: *H. musciformis* EtOAc fraction (0.51) > *K. alvarezii* EtOAc fraction (0.84) > *J. rubens* DCM fraction (1.08) > *H. valentiae* EtOAc fraction (1.1). As a whole, the $ABTS^{\cdot+}$ scavenging capacities of the EtOAc fraction of *H. musciformis* and *K. alvarezii* was stronger than MeOH extracts and fractions of other studied seaweeds. The ABTS.+ scavenging activity was found to be positively correlated with each other as well as TPC ($r^2 = 0.235$) (Fig. 6.12B) thereby realizing the role of phenolic compounds responsible for the antioxidant properties of the seaweed extracts.

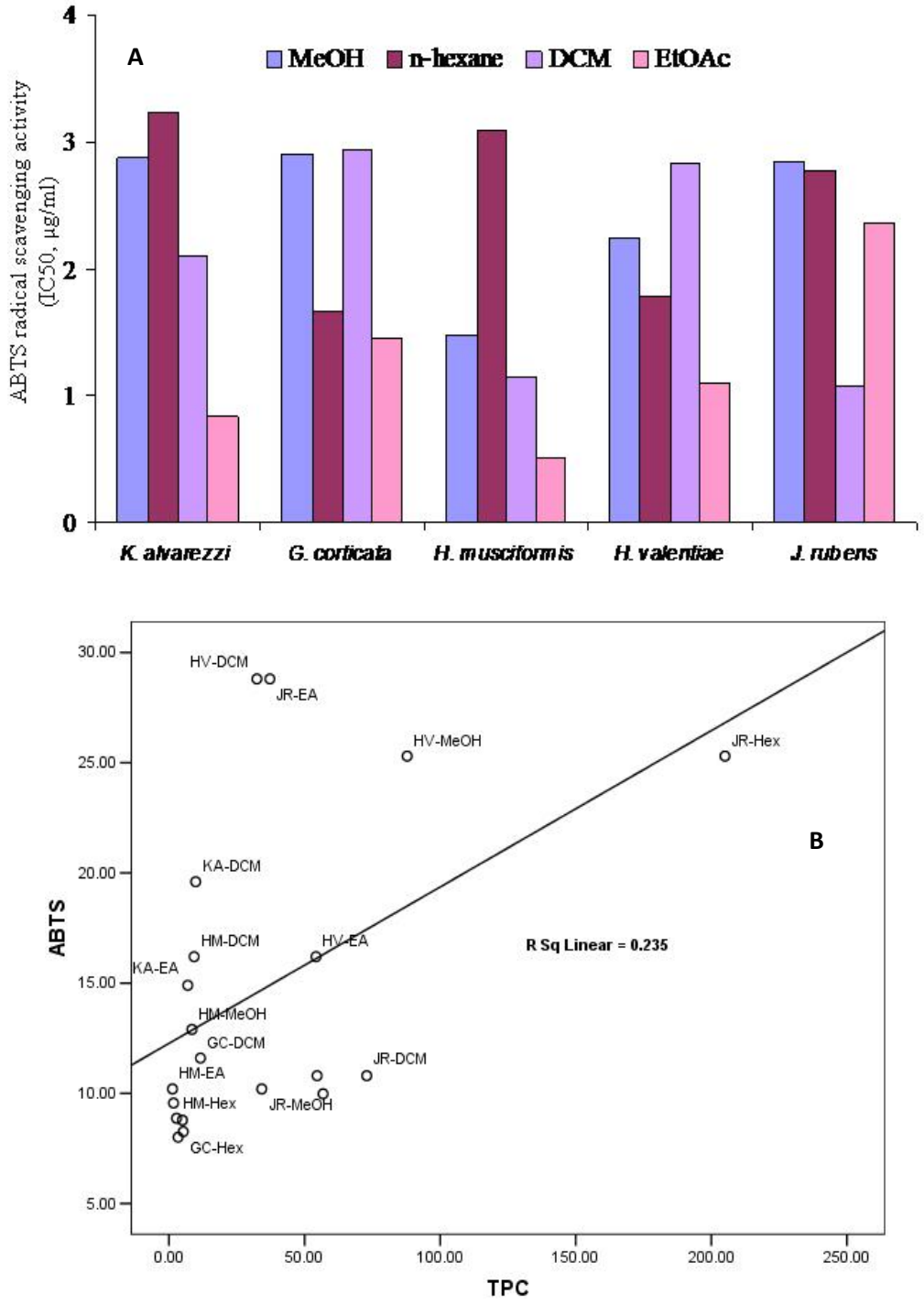


Fig. 6.12. **(A)**IC_{50} values for $ABTS^{\cdot+}$ scavenging activities (µg/ml) and (**B**) Scatter plot diagram showing the correlation of Total phenolic content & $ABTS^{\cdot+}$ scavenging activities of different seaweeds

6.2.2.2.3. Hydroxyl Radical Scavenging Activity

Hydroxyl radical scavenging activity was employed to understand the potential of different seaweed extracts/fractions against short-lived radicals, *viz*., $HO^{\cdot}$ radical that abstract H atoms, which causes peroxide reaction of lipids. $HO^{\cdot}$ radical scavenging activities of the MeOH extracts/fractions of the red seaweeds at 0.6 mg/ml are recorded in Table 6.1. $HO^{\cdot}$ scavenging activity of MeOH extracts was in the order: *J. rubens* (45.3 %) > *H. valentiae* (37.0 %) > *K. alvarezii* (16.2 %) > *H. musciformis* (15.8 %) > *G. corticata* (13.1 %). DCM fractions of all the red seaweeds except *K. alvarezii* registered significantly higher ($p<0.05$) $HO^{\cdot}$ radical scavenging activity (35.4 - 51.2 %) than other solvent fractions as evident from Table 6.1. Interestingly, the IC_{50} values (Fig. 6.13A) also established the maximum $HO^{\cdot}$ scavenging effect for the DCM fractions of red seaweeds except *K. alvarezii* ($IC_{50} \leq$ 0.96). The present study correlates well with earlier studies that >90 % HO. scavenging activity was reported from DCM and BuOH fractions (1 mg/ml) of red seaweeds *Acanthophora spicifera* and *Gracilaria edulis* (Ganesan *et al*., 2008). The extracts of red seaweeds, *Gracilaria verrucosa*, *G. textorii*, *Grateloupia filicina* and *Polysiphonia japonica* also reported potentially high HO. scavenging activity (Heo *et al*., 2006). $HO\cdot$ scavenging activity was found to be positively correlated with each other as well as TPC ($r^2 = 0.009$) (Fig.6.13B) which indicated the effect of phenolic compounds on the scavenging of $HO^{\cdot}$ radicals.

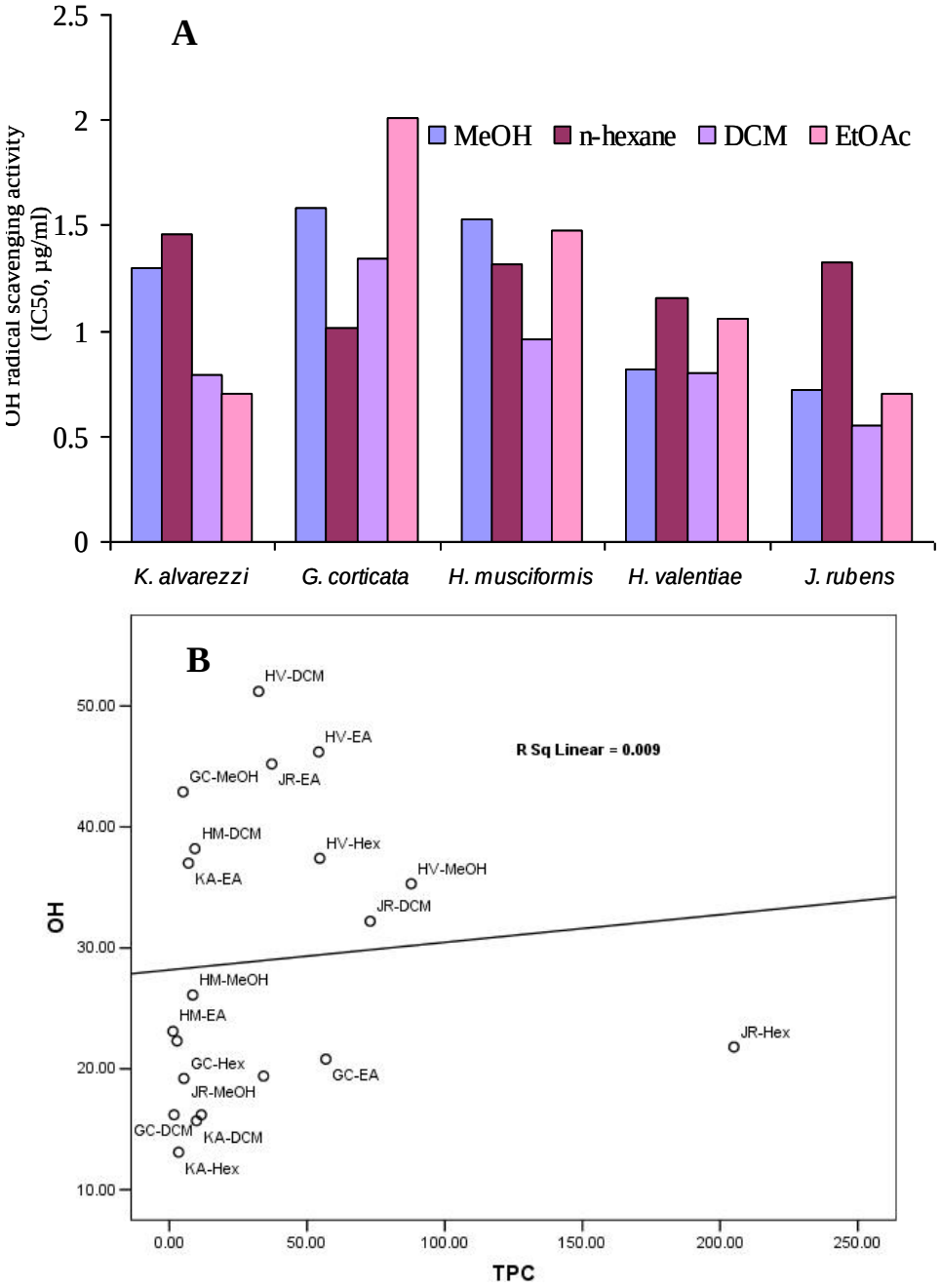


Fig. 6.13. **(A)**IC_{50} values for HO· scavenging activity (mg/ml) and (**B**) Scatter plot diagram showing the correlation of Total phenolic content & HO· scavenging activity of different seaweeds

6.2.2.2.4. *Scavenging of Hydrogen Peroxide*

H_2O_2, a reactive non-radical compound, is of potential biological significance because of its ability to penetrate biological membranes. H_2O_2 itself is not very

reactive, but it may convert into more reactive species (singlet oxygen and HO. radicals) (Ruch *et al*., 1989). H_2O_2 scavenging activity (%) at 1 mg/ml of the MeOH extracts/fractions of the red seaweeds is recorded in Table 6.1. EtOAc and DCM fractions of *H. musciformis* registered significantly higher ($p<0.05$) H_2O_2 scavenging activity (80.8 & 75.5 %, respectively) than its MeOH extract (43 %), *n*-hexane fraction (32.9 %). Although *K. alvarezii* EtOAc fraction showed significantly lower H_2O_2 scavenging activity (70.8 %) compared to that of *H. musciformis* EtOAc fraction (80.8 %) ($p<0.05$), it showed significantly higher H_2O_2 scavenging activity than *G. corticata*, *J. rubens* and *H. valentiae* EtOAc fractions ($p<0.05$). According to the IC_{50} values showed in Fig. 6.14A, theH_2O_2 scavenging activity of the EtOAC fraction of five seaweeds exhibited the order: *H. musciformis* (0.39) > *K. alvarezii* (0.44) > *J. rubens* (0.57) > *H. valentiae* (0.64) > *G. corticata* (0.87). The significantly higher H_2O_2 scavenging effect of the EtOAc fractions can be explained due to the presence of hydrophilic phenolics (Senevirathne *et al*., 2006). The EtOAc fraction of the red seaweed *Ecklonia cava* (Heo *et al*., 2006) reported higher H_2O_2 scavenging activity as supporting our observations that red seaweeds are rich source of natural antioxidant compounds, capable to scavenge H_2O_2. H_2O_2 scavenging activity was found to be positively correlated with each other as well as TPC (r^2 = 0.467) (Fig.6.14B) thereby realizes the role of phenolic compounds responsible for the antioxidant properties of seaweed extracts. Although H_2O_2 is not a highly reactive molecule, it can sometimes be toxic to body cells or food systems because it may give rise to $HO^{\cdot}$ radicals and singlet oxygen by reacting with transition metal ions (Halliwell & Gutteridge 1999). It is therefore, the scavenging of H_2O_2 by natural antioxidant sources is important for the protection of biological systems.

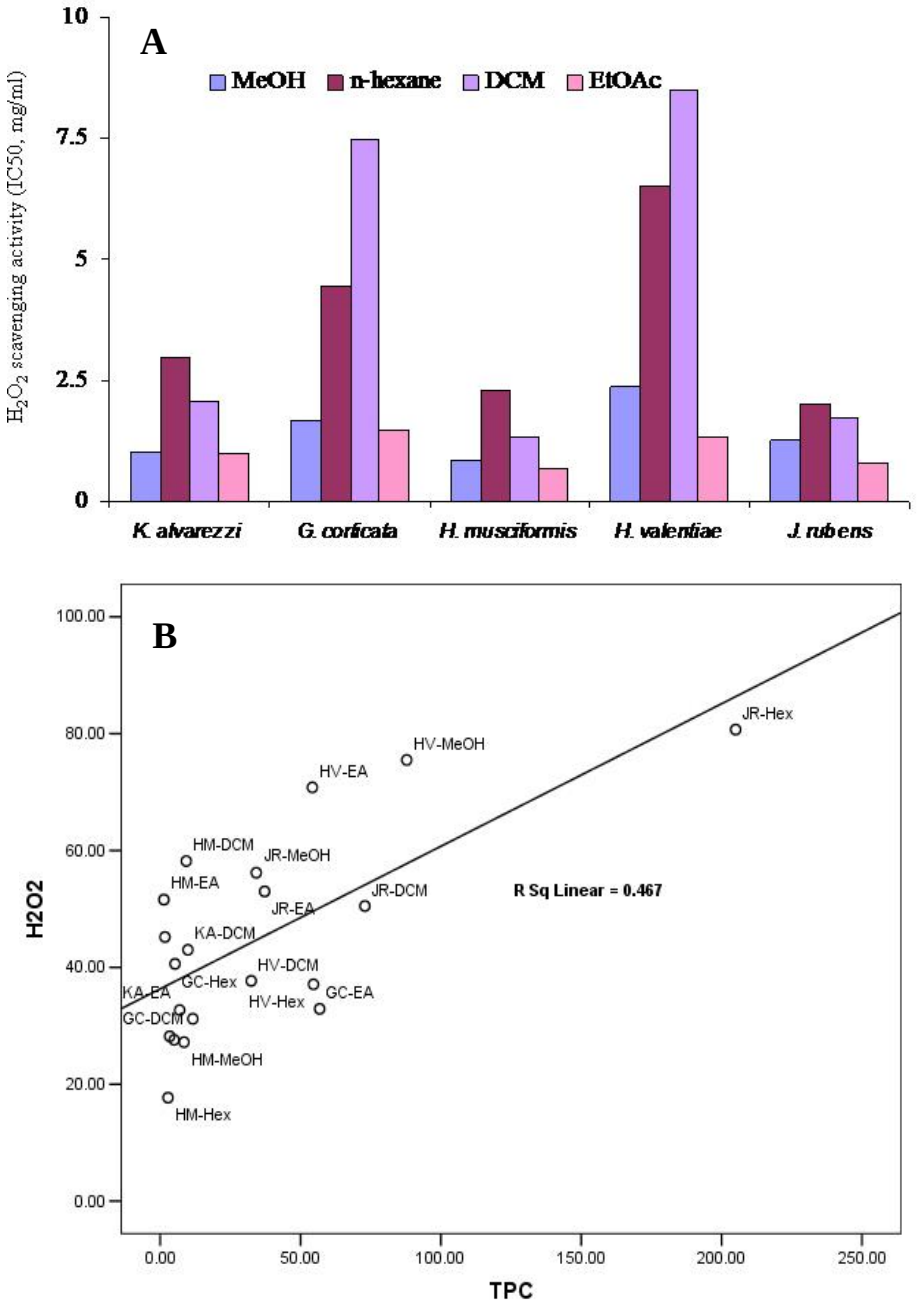


Fig. 6.14. (**A**)IC_{50} values for H_2O_2 scavenging capacity (mg/ml) and (**B**) Scatter plot diagram showing the correlation of Total phenolic content & H_2O_2 scavenging capacity of different seaweeds

6.2.2.2.5. Lipid Peroxidation Inhibition Ability: Thiobarbituric Acid-Reactive Substances (TBARS) Formation Inhibitory Activity

TBARS assay, which reflect the production of low molecular weight end products, like malondialdehyde is used to indicate free-radical generation. TBARS formation inhibitory activity at 2 mg/ml of the MeOH extracts/fractions of the five red

seaweeds was recorded in Table 6.1. EtOAc (2.7 - 3.9 MDAEQ/ kg) and DCM (3.6 - 9.8 MDAEQ/kg) fractions of all five seaweeds exhibited significantly higher TBARS formation inhibitory ability than its corresponding MeOH extract (9.9 - 18.7 MDAEQ/kg) and *n*-hexane fractions (10.3 - 18.0 MDAEQ/kg) ($p<0.05$). The present study correlates well with the earlier studies (Zubia *et al*., 2009) reporting that EtOAc and DCM fractions are the major seaweed fractions harboring the principle antioxidative components that inhibits lipid peroxidation compared to *n*-hexane fraction and total MeOH extract. Apparently in the present study, EtOAc fraction of *H. musciformis* registered significantly higher TBARS inhibition ability (2.71 MDAEQ/kg) followed by *K. alvarezii* > *J. rubens* > *G. corticata* > *H. valentiae*. The inhibition of lipid peroxidation may be due to the presence of polyphenolic antioxidants that were reported to disrupt free-radical chain reaction by donating a proton to fatty acid radicals to terminate chain reactions (Karawita *et al*., 2005). However, a negative correlation was realized between TPC and lipid peroxidation inhibition activity ($r^2 = 0.318$) (Fig. 6.15), which indicate that the antioxidant activity did not depend only on TPC, but also on other factors. The bioactive metabolites other than phenolics such as polysaccharides might play an important role to inhibit capable of inhibiting the TBA-MDA adduct formation during the lipid oxidation process (Muzzarelli 1997).

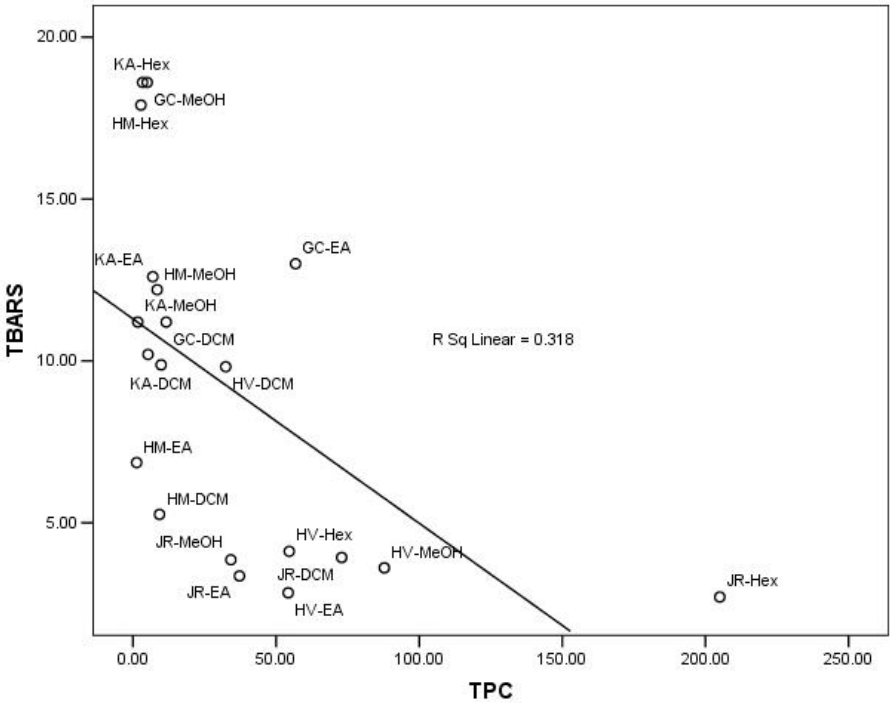


Fig. 6.15. Scatter plot diagram showing the correlation of Total phenolic content & TBARS formation inhibitory activity of different seaweeds

6.2.2.2.6. Total Reduction Capability

Fe^{3+} reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action, and can be strongly correlated with other antioxidant properties (Dorman *et al*., 2003). Total reduction capability at highest concentration (1 mg/ml) of the MeOH extracts/fractions of the five red seaweeds are recorded in Table 6.1. Significantly higher total reduction capability was observed for MeOH extracts/fractions of *H. musciformis* (Abs_{700nm} 0.74 - 1.46) compared with other seaweed extract/fractions. EtOAc fraction of *H. musciformis* (Abs_{700nm} 1.46) showed maximum reducing power followed by the similar fractions of *K. alvarezii*, *H. valentiae*, *J. rubens* and *G. corticata* (Abs_{700nm} 0.96, 0.48, 0.45 & 0.32, respectively). Our study is in accordance with the earlier reports which reported that the reducing power of MeOH ($Abs_{700\ nm}$ 0.07 - 0.74) and EtOAc extracts (Abs_{700nm} 0.013–0.467) of red seaweed *Kappaphycus alvarezii* extracts were reported to be higher than *n*-hexanic extract (Abs_{700nm} 0.017–0.16 at 0.5–5 mg/ml) (Kumar *et al*., 2008). Furthermore, EtOAc fractions of red seaweed *Rhodomela confervoides* exhibited potentially high reducing power (426 mg/g ascorbic acid equivalents) (Wang *et al*., 2009). Generally, the reducing properties are associated with the presence of compounds, which exert their action by breaking the free radical chain by donating a hydrogen atom or a single electron (Wong *et al*., 2006). The total reduction capability was found to be positively correlated with total phenolics (r^2 = 0.503) (Fig. 6.16) supporting the former statements on the contribution of phenolic compounds in the antioxidant activities.

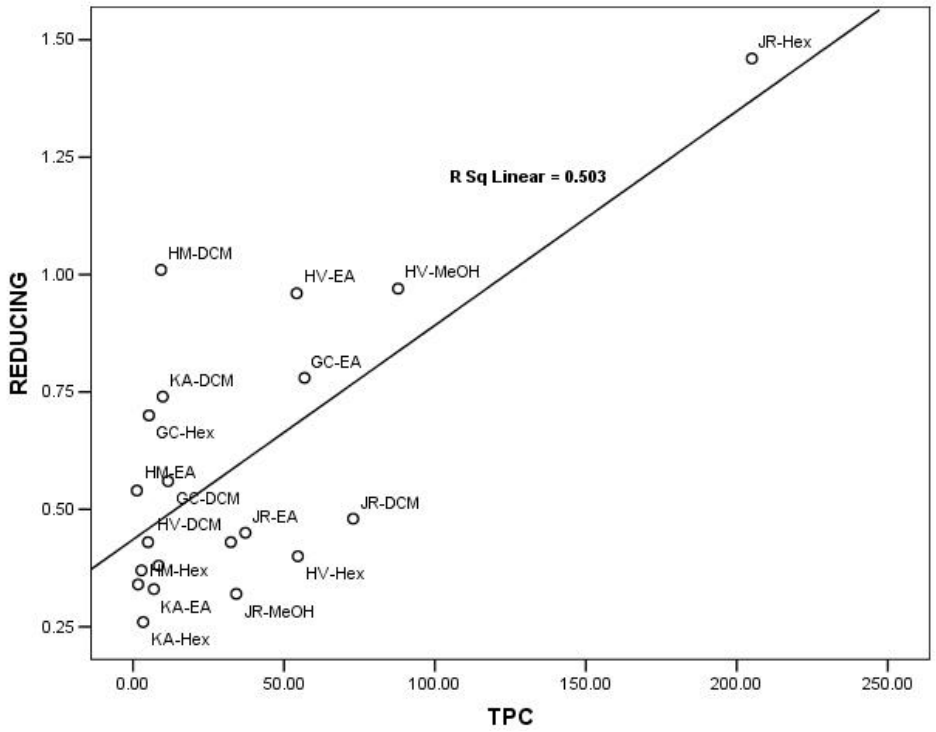


Fig. 6.16. Scatter plot diagram showing the correlation of Total phenolic content & total reduction capability of different seaweeds

6.2.2.2.7. Fe^{2+} ion Chelating Activity

Metal chelating capacity was significant since it reduced the concentration of the catalyzing transition metal in lipid peroxidation. Fe^{2+} ion chelating activity (%) at 0.6 mg/ml of the MeOH extracts of the five red seaweeds (Table 6.1) were in the order: *H. musciformis* > *J. rubens* > *K. alvarezii* > *G. corticata* > *H. valentiae*. EtOAc extracts of *H. musciformis* and *J. rubens* were better chelators of ferrous ion (38.2 & 22.7 %, respectively) compared with *K. alvarezii* and *H. valentiae* (20.3 & 18.8 %). These results are in accordance with earlier studies that demonstrated that polyphenols derived from seaweeds are potent Fe^{2+} chelators (Senevirathne *et al*., 2006), and metal chelating potency of phenolic compounds are dependent upon their unique phenolic structure, and the number and location of –OH groups (Lindsay 1996). Interestingly, according to the IC_{50} values (Fig. 6.17A), the EtOAc fractions of five seaweeds exhibited strong chelating activity (*H. musciformis* > *J. rubens* > *K. alvarezii* > *H. valentiae* > *G. corticata*) followed by MeOH extracts. The TPC and Fe^{2+} ion chelating activity exhibited a positive correlation (r^2 = 0.254) (Fig.6.17B)

thus suggesting that phenolics capable of chelating transition metals are present in these red seaweeds. Generally, the compounds with structures containing two or more of the following functional groups: –OH, –SH, –COOH, $–PO_3H_2$, >C=O, $–NR_2$, –S– and –O– in a favorable structure-function configuration were reported to have chelation activity (Lindsay 1996).

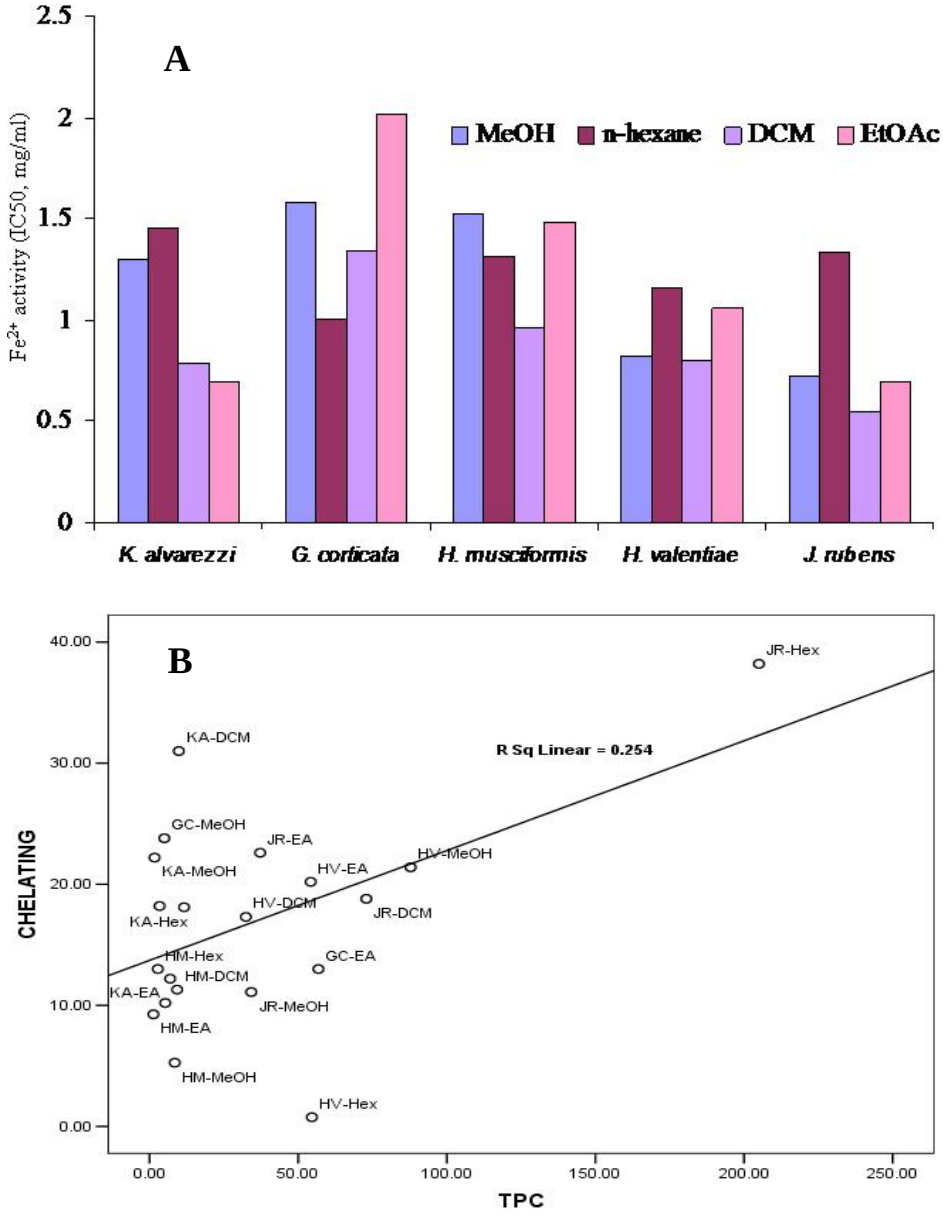


Fig. 6.17. (**A**) IC_{50} values for Fe^{2+} ion chelating activity (mg/ml) and (**B**) Scatter plot diagram showing the correlation of Total phenolic content & Fe^{2+} ion chelating activity (mg/ml) of different seaweeds

Table 6.1. Total phenolic content (mg GAE/g sample), ABTS, DPPH, ·OH, H_2O_2 radical scavenging activities (%), lipid peroxidation inhibitory activity (MDAEQ/kg), total reduction capability (Absorbance at 700 nm) and Fe^{2+} ion chelating activity (%) of the crude methanolic extracts and solvent fractions of the candidate red seaweeds

Seaweeds	MeOH extracts	Solvent fractions		
		n-hexane	DCM	EtOAc
Total phenolic content (5mg/ml)				
K. alvarezzi	1.67 ± 0.17^{ap}	5.26 ± 0.53^{ap}	9.26 ± 0.93^{bp}	54.26 ± 5.43^{cp}
G. corticata	3.36 ± 0.34^{ap}	11.67 ± 1.17^{aq}	1.29 ± 0.13^{aq}	34.23 ± 3.42^{bq}
H .musciformis	9.84 ± 0.03^{aq}	56.81 ± 2.4^{br}	87.82 ± 3.90^{cr}	205.48 ± 2.40^{dr}
H. valentiae	6.91 ± 0.06^{apq}	8.46 ± 0.18^{apq}	54.68 ± 0.19^{bs}	72.95 ± 1.25^{cs}
J. rubens	4.95 ± 0.06^{ap}	2.77 ± 0.02^{ap}	32.48 ± 1.04^{bt}	37.27 ± 0.72^{bq}
DPPH· radical scavenging activity (1 mg/ml)				
K. alvarezzi	11.02 ± 1.1^{apr}	2.25 ± 0.23^{bp}	20.56 ± 2.06^{cp}	61.29 ± 6.13^{dp}
G. corticata	6.54 ± 0.65^{aq}	1.56 ± 0.16^{ap}	31.01 ± 3.1^{bq}	18.45 ± 1.85^{cq}
H. musciformis	15.40 ± 1.05^{ap}	24.93 ± 0.66^{bq}	69.42 ± 1.04^{cr}	82.98 ± 0.18^{dr}
H. valentiae	7.73 ± 0.07^{aqr}	2.94 ± 0.586^{ap}	66.36 ± 2.81^{br}	20.53 ± 0.86^{cq}
J .rubens	17.69 ± 2.44^{ap}	14.97 ± 0.33^{ar}	40.38 ± 1.46^{bs}	29.93 ± 0.27^{cs}
ABTS radical scavenging activity (0.6 µg/ml)				
K. alvarezzi	9.56 ± 0.96^{ap}	8.26 ± 0.83^{ap}	16.24 ± 1.62^{bp}	39.26 ± 3.93^{cp}
G. corticata	8.01 ± 0.8^{ap}	11.6 ± 1.16^{ap}	10.26 ± 1.03^{ap}	22.16 ± 2.22^{bq}
H. musciformis	19.60 ± 1.44^{aq}	9.98 ± 0.73^{bp}	25.39 ± 0.55^{cq}	63.30 ± 1.73^{dr}
H. valentiae	14.96 ± 0.42^{apq}	12.90 ± 0.64^{ap}	10.89 ± 1.02^{ap}	27.90 ± 0.2^{bq}
J. rubens	8.78 ± 0.30^{ap}	8.87 ± 0.31^{ap}	28.86 ± 0.30^{bq}	11.09 ± 1.16^{cs}
Hydroxyl radical scavenging activity (0.6mg/ml)				
K. alvarezzi	16.21 ± 1.62^{ap}	19.26 ± 1.93^{ap}	38.26 ± 3.83^{bp}	46.25 ± 4.63^{cp}
G. corticata	13.14 ± 1.31^{ap}	16.21 ± 1.62^{abp}	23.16 ± 2.32^{bq}	19.46 ± 1.95^{bq}
H. musciformis	15.77 ± 0.32^{ap}	20.87 ± 0.11^{bp}	35.39 ± 0.10^{cp}	21.8 ± 1.11^{bq}
H. valentiae	37.03 ± 1.47^{aq}	26.14 ± 1.16^{bq}	37.42 ± 1.42^{ap}	32.27 ± 1.44^{ar}
J rubens	42.99 ± 1.26^{aq}	22.31 ± 0.61^{bpq}	51.20 ± 1.45^{cr}	45.29 ± 1.89^{ap}
H_2O_2 scavenging capacity (1 mg/ml)				
K. alvarezzi	45.21 ± 0.52^{ap}	40.6 ± 0.06^{bp}	58.28 ± 0.83^{cp}	70.81 ± 7.08^{dp}
G. corticata	28.26 ± 2.83^{aq}	31.2 ± 3.12^{aq}	51.64 ± 5.16^{bp}	56.25 ± 5.63^{bq}
H .musciformis	43.01 ± 0.81^{ap}	32.93 ± 0.56^{bq}	75.54 ± 0.72^{cq}	80.77 ± 0.60^{cp}
H .valentiae	32.75 ± 1.03^{aq}	27.28 ± 1.26^{aq}	37.16 ± 0.83^{ar}	50.5 ± 0.72^{bq}
J. rubens	27.63 ± 1.36^{aq}	17.74 ± 1.06^{br}	37.78 ± 0.58^{cr}	53.07 ± 1.06^{dq}
Lipid peroxidation inhibitory activity (2 mg/ml)				
K. alvarezzi	11.26 ± 1.13^{ap}	10.26 ± 1.03^{ap}	5.26 ± 0.53^{bp}	2.84 ± 0.28^{bp}
G. corticata	18.68 ± 1.87^{aq}	11.26 ± 1.13^{bp}	6.86 ± 0.69^{cp}	3.86 ± 0.39^{cp}
H musciformis	9.88 ± 0.24^{ap}	13.05 ± 0.08^{ap}	3.61 ± 0.37^{bp}	2.71 ± 0.10^{bp}
H. valentiae	12.68 ± 0.48^{ap}	12.28 ± 0.74^{ap}	4.12 ± 0.16^{bp}	3.93 ± 0.15^{bp}
J. rubens	18.60 ± 2.40^{aq}	17.99 ± 1.77^{ap}	9.82 ± 0.26^{bp}	3.36 ± 0.10^{cp}

Total reduction capability (1 mg/ml)				
K. alvarezzi	0.34±0.03^{ap}	0.7±0.07^{ap}	1.01±0.1^{ap}	0.96±0.1^{ap}
G. corticata	0.26±0.03^{ap}	0.56±0.06^{ap}	0.54±0.05^{ap}	0.32±0.03^{ap}
H. musciformis	0.74 ± 0.01^{ap}	0.78 ± 0.01^{ap}	0.97 ± 0.02^{ap}	1.46 ± 0.02^{ap}
H valentiae	0.33 ± 0.03^{ap}	0.38 ± 0.01^{ap}	0.40 ± 0.01^{ap}	0.48 ± 0.01^{ap}
J .rubens	0.43 ± 0.01^{ap}	0.37 ± 0.01^{ap}	0.43 ± 0.01^{ap}	0.45 ± 0.01^{ap}
Fe^{2+} ion chelating activity (0.6mg/ml)				
K. alvarezzi	22.21±2.22^{ap}	10.26±1.03^{bp}	11.31±1.13^{bp}	20.27±2.03^{ap}
G. corticata	18.26±1.83^{ap}	18.16±1.82^{aq}	9.26±0.93^{bp}	11.16±1.12^{bq}
H. musciformis	31.02 ± 0.98^{aq}	13.04 ± 0.32^{bp}	21.42 ± 0.95^{cq}	38.29 ± 1.7^{dr}
H. valentiae	12.26 ± 0.27^{ar}	5.26 ± 0.20^{br}	0.76± 0.51^{cr}	18.84 ± 0.31^{dp}
J. rubens	23.87± 1.41^{ap}	13.06 ± 0.72^{bp}	17.33 ± 0.64^{bq}	22.67 ± 1.60^{ap}

Data are the mean values of triplicate and expressed as mean ± standard deviation. $^{a–c}$ Row wise values with different superscripts of this type indicate significant difference ($p<0.05$). $^{p–s}$ Column wise values with different superscripts of this type indicate significant difference ($p<0.05$). DCM - Dichloromethane, EtOAc - Ethyl acetate. Concentration of samples for each analysis is given in parentheses.

6.2.3. Natural Herbs - Rosemary and Green Tea

6.2.3.1. HPLC Profiling of the Rosemary and Green Tea Extracts

The HPLC profile of the rosemary extracts showed that the principal antioxidative components of rosemary extracts are the phenolic diterpene, carnosic acid (the major compound) (Fig. 6.18A). In earlier studies, rosemary extracts were shown to have similar phenolic compound and their antioxidant activity was attributed mainly to their carnosic acid, carnosol and rosmarinic acid components (Erkan *et al*., 2008).

The HPLC profile of the green tea extracts showed that it contains flavanols *viz*. catechins - epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epicatechin (EC), catechin gallate and catechin (Fig. 6.18B). Earlier studies showed the presence of epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG) as green tea polyphenols and among them, EGCG was found to be the most abundant and biologically active compound (Vinson *et al*., 1995).

The Fig. 6.19A&B shows the chemical structures of the compounds., carnosic acid, carnosol, epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epicatechin (EC), catechin gallate and catechin.

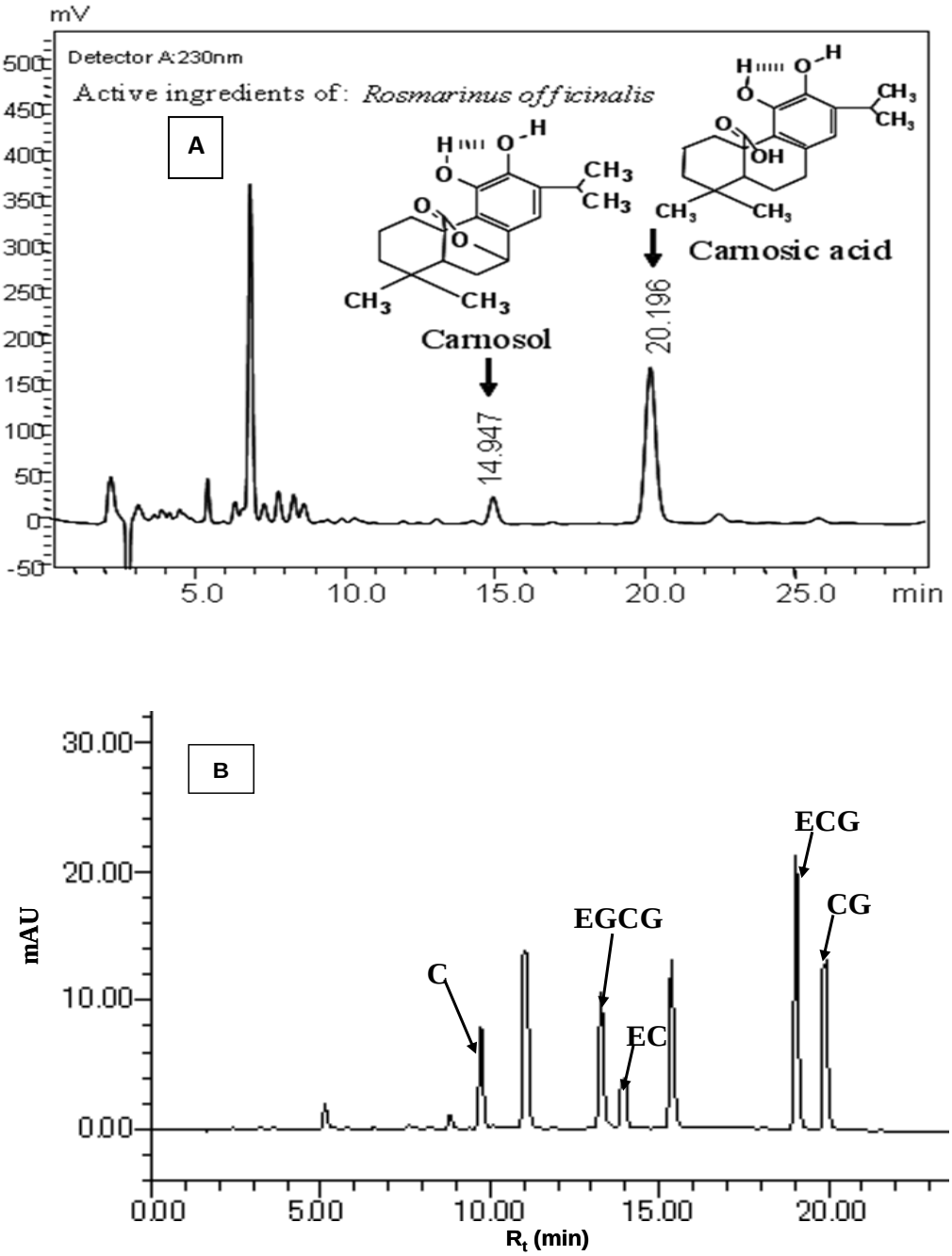


Fig. 6.18. Chromatograms of (**A**) rosemary extract showing the peak of carnosol & carnosic acid and (**B**) green tea extract showing the peaks of (-)catechin C, (-)epigallocatechin gallate EGCG, (-)epicatechin EC, (-) epicatechin gallate ECG & (-)catechin gallate CG

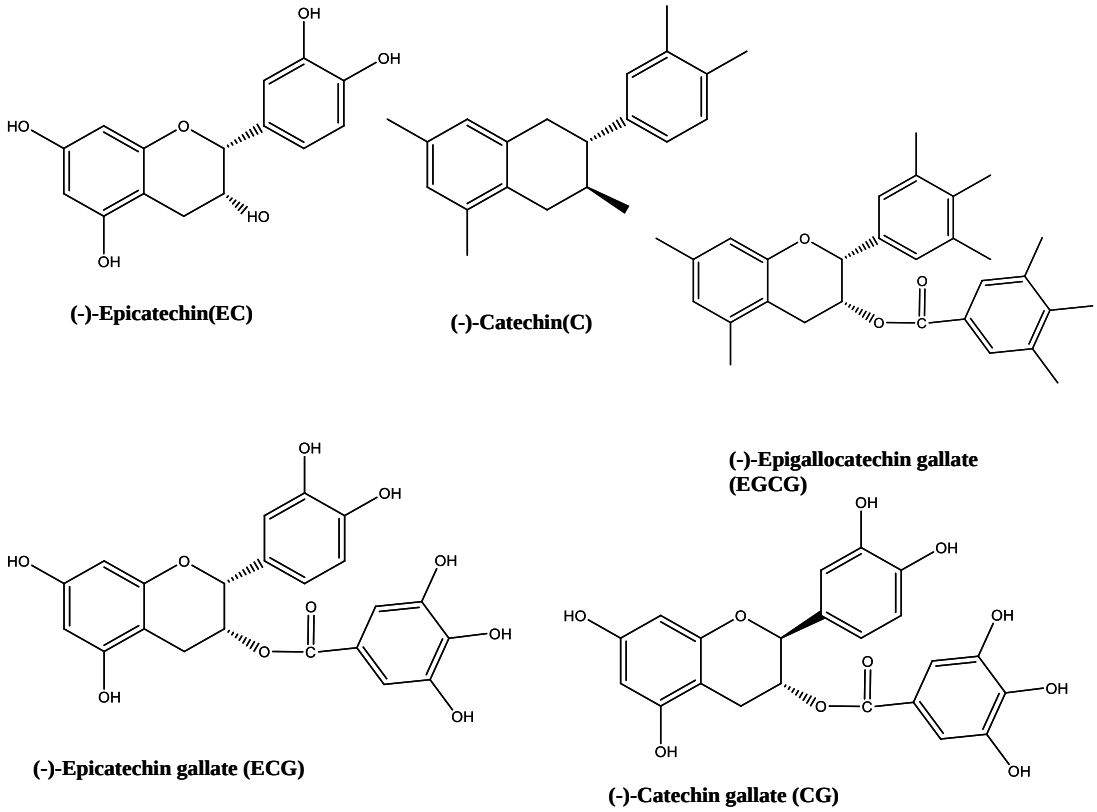


Fig.6.19. Chemical structures of the compounds in green tea extract; epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epicatechin (EC), catechin gallate and catechin.

6.2.3.2. Antioxidant Activities

6.2.3.2A. Total Phenolic Content of Rosemary and Green Tea Extracts

The total phenolic content (TPC) observed for rosemary and green tea extracts and their different combinations are shown in Fig. 6.20A. The green tea extract showed significantly higher total phenolic content (368.5 mg GAE/g) compared with rosemary extract (271.2 mg GAE/g). The combination rosemary: green tea 20:80 (%,w/w) showed maximum total phenolic content (381.5 mg GAE/g) followed by the combinations of rosemary and green tea extracts in the ratios 20:80, 60:40, 80:20, 50:50 and 40:60 (%, w/w; respectively). The high phenolic contents further showed the presence of phenolic compounds such as carnosic acid, carnosol and rosmarinic acid and catechin components in these extracts.

6.2.3.2B. 1, 1-Diphenyl-2-Picryl-Hydrazil (DPPH•) Scavenging Activity

DPPH radical scavenging activities (%) of the extracts/ combinations were found to be maximum at 48th h. The scavenging activity observed at 0.5 mg/ml (48th

h) is shown in Fig. 6.20B. Green tea and rosemary extracts showed similar DPPH radical scavenging activity (91 %). However among different combinations of these extracts, rosemary: green tea used at 60:40 (% w/w) showed maximum DPPH radical scavenging activity compared with other combinations (%, w/w) ($p>0.05$). The enhanced effect of the combinations could be attributed to its higher number of hydroxyl groups that were able to scavenge more free radicals.

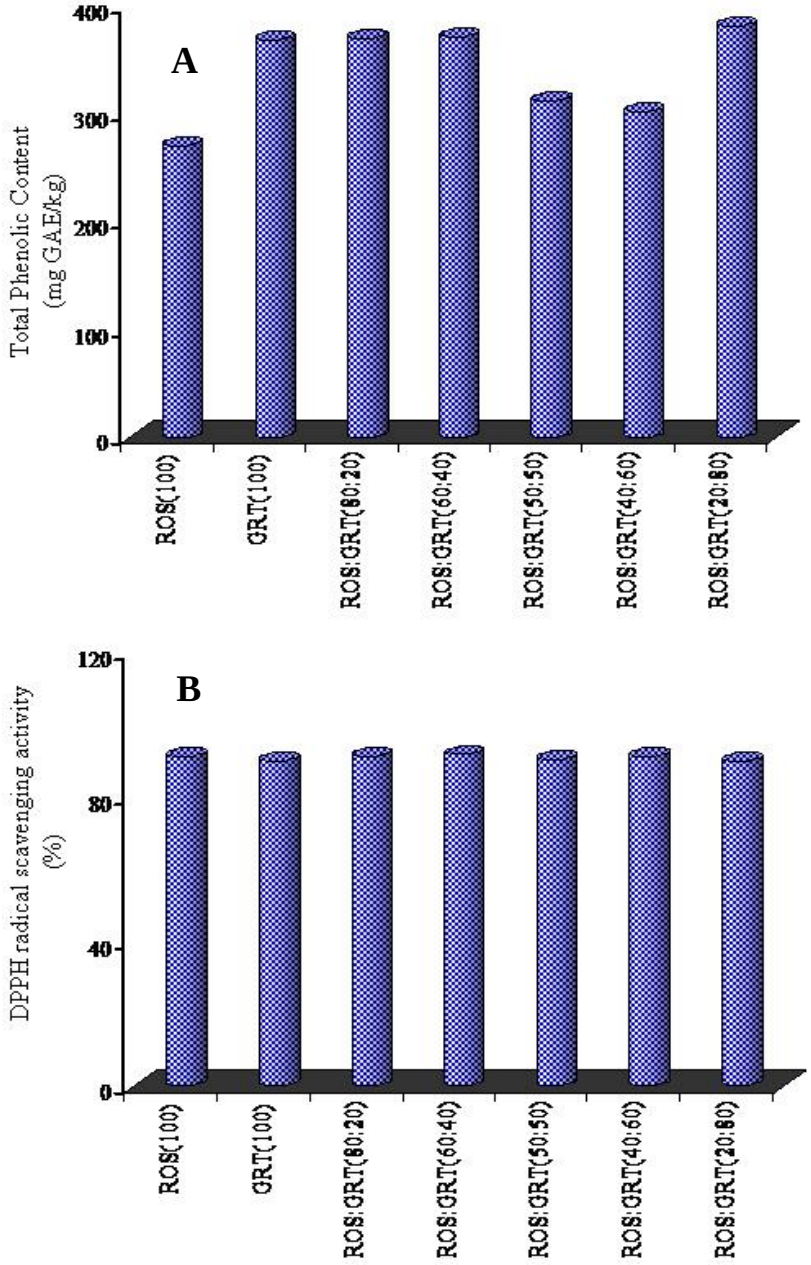


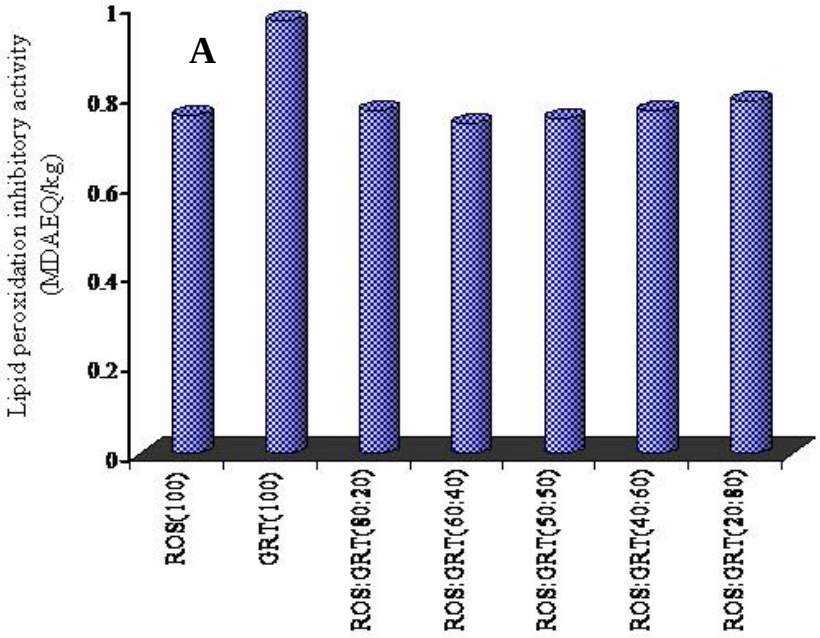
Fig. 6.20. (**A**) Total phenolic content (mg GAE/g); (**B**) DPPH· scavenging activity (%) of rosemary and green tea extracts and their different combinations at the ratios 80:20, 60:40, 50:50, 40:60 and 20:80 (% w/w).

6.2.3.2C. Thiobarbituric Acid-Reactive Substances (TBARS) Inhibitory Activity

TBARS assay, which reflect the production of low molecular weight end products, like malondialdehyde is used to indicate free-radical generation. TBARS formation inhibitory activity at 2 mg/ml of the green tea and rosemary extracts/combinations was recorded in Fig. 6.21A. TBARS inhibitory activity was found to be more for rosemary extract (0.76 MDAEQ/kg) as compared to the green tea extract (0.97 MDAEQ/kg). Higher TBARS inhibitory activity was observed for the combination, rosemary:green tea 60:40 (% w/w) (0.74 MDAEQ/kg) than others. A previous study using the green tea and rosemary extract combinations also supported their effect on reducing the TBARS values (Mohamed 2007).

6.2.3.2D. Fe^{2+} ion Chelating Activity

Fe^{2+} ion chelating activity at 0.6 mg/ml of the green tea and rosemary extracts/combinations was recorded in Fig. 6.21B. The Fe^{2+} ion chelating activity was found to be more for rosemary extract (56.3 %) compared to green tea extract (66.3 %). The maximum Fe^{2+} ion chelating activity was observed for the rosemary:green tea 60:40 (% w/w) combination (69.5 %) as compared with other combinations. Catechins and other phenolic compounds had recognized as efficient antioxidants for chelating metal ions (Wanasundara & Shahidi 1998).



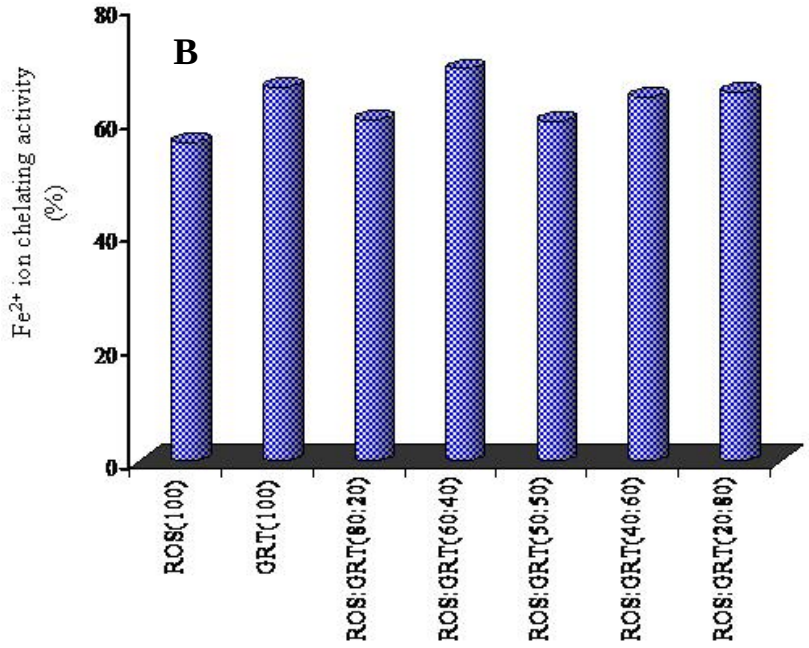


Fig. 6.21. **(A)** TBARS formation inhibitory activity (MDAEQ/kg) and **(B)** Fe^{2+} ion chelating activity (%) of rosemary and green tea extracts and their different combinations at the ratios 80:20, 60:40, 50:50, 40:60 and 20:80 (% w/w).

6.3. Conclusions

The present chapter provides information regarding the potential of halophytes and red seaweeds to develop natural sources for antioxidants as food supplements, as nutraceuticals and/or functional foods, and candidates in combating carcinogenesis and inflammatory diseases. The significant positive correlation of total phenolic content with different antioxidant activities indicates that phenolic principles present in these marine additives are endowed with potential antioxidant properties. A blend prepared from the EtOAc extracts of the halophytes, *S. brachiata* and *S. maritima* were observed to possess high antioxidant potential as compared to other halophytes when their *in vitro* antioxidant properties were considered. Similarly, the EtOAc fractions obtained from the crude methanolic extracts of the red seaweeds, *K. alvarezii*, *H. musciformis* and *J. rubens* revealed higher overall antioxidant potential as compared to *G. corticata* and *H. valentiae*. However, *H. valentiae* showed higher content of total phenolics as compared to *J. rubens* and *K. alvarezii* and the former it also showed better ABTS radical scavenging activity than

that of *J. rubens.* Furthermore, *H. valentiae* also exhibited higher hydroxyl radical scavenging activity than *H. musciformis*. Similarly, *G. corticata* showed better ABTS radical scavenging activity as compared to *H. musciformis*. It is therefore, that these five candidate seaweeds along with the two halophytic plant extracts, *S. brachiata* and *S. maritima*, were selected to assess the effect of these marine natural additives on lipid oxidation in various forms of fish oil in the following chapter (*Chapter 7*).

Chapter 7

STABILITY STUDIES OF SARDINE OIL

Contents

Background

Fish oils, rich in polyunsaturated fatty acids, are very sensitive to oxidation due to the presence of multiple methylene interrupted double bonds. The traditional methods to reduce lipid oxidation utilize synthetic phenolic antioxidants such as BHA, BHT and propyl gallate. In order to maintain the quality of the sardine fish oil the marine extracts shortlisted in *Chapter 6* were evaluated for their effects on oxidative stability of the oils. Unlike the synthetic antioxidants, these natural bioactive extracts may be added in higher quantities as they comprise only natural compounds, and it is likely that these would be granted generally recognized as safe (GRAS) status. In the current study, the marine extracts were added at a rate of 0.5 % (w/w of oil).

The purpose of the present study was to evaluate the protective effects of different marine derived natural antioxidant extracts (0.5 %) as compared with that of BHT and α-tocopherol (0.1%) in refined oil (RO, *Chapter 4*) or concentrated fatty acid methyl esters (CO, *Chapter 5*) produced from oil sardines. The three most active additives (extracts) were then tested in different combinations to the concentrated fatty acid methyl esters for studying the synergistic effect of these extracts. The optimal concentrations of the potent combinations were selected by further studying their effectiveness to prevent oxidation of the purified fatty acid methyl esters (PO). The purified fatty acid methyl esters protected with marine additives was finally compared to the fatty acid methyl esters protected with natural antioxidant blend. Accelerated conditions of oxidation were used to assess the stability by using the rancimat method, which was performed at 80 ^{o}C and under accelerated conditions at 65 ^{o}C in an oven for 12 days. Tests of oxidative stability are

commonly used to evaluate the shelf life of fish oils/esters, but in order to reduce the time required to complete the study, an elevated temperature (65 ^{o}C) was used to accelerate the oxidation process. The objective of this accelerated shelf life study was to predict the shelf life of the sardine oil/methyl esters stored at ambient temperatures. The monitorization of the oxidative indices (P.V., pA.V., TOTOX, TBARS values and DPPH activity) were also performed at various intervals during the experimental storage period.

7.1. Materials and Methods

7.1.1. Chemicals, Reagents and Instrumentation

The solvents and reagents used for sample preparation were of analytical grade (E-Merck, Darmstadt, Germany). Analytical grade solvents were redistilled in an all-glass system. The glassware was rinsed with $CHCl_3$-CH_3OH (2: l, v/v), and dried under N_2. Other chemicals and reagents are as explained in the previous chapters.

GLC data were recorded on a Perkin-Elmer AutoSystem XL gas chromatograph. The spectrophotometric measurements were performed using UV-visible spectrophotometer (Varian Cary 50, USA). FT-IR analysis was carried out by the potassium bromide (KBr) pellet method, and was recorded on Fourier transform-infrared spectrometer (Shimadzu, 8400S) with a high sensitivity pyroelectric detector in a range of 7800 – 350 cm^{-1}. ^{1}HNMR spectra of the purified fatty acid methyl esters were measured on BRUKER (AVANCE III, 400 MHz) NMR spectrometer. The distillation under reduced pressure was performed using a rotary vacuum evaporator (Heidolph, Germany; Hei-VAP series). The samples for shelf life studies were kept in an incubator (Labline, India). A Metrohm Rancimat model 743 (Methrom Instruments, Herisau, Switzerland) was utilized for studying the stability.

7.1.2. Experimental Design of Stability Studies

In order to find the potent antioxidant additives the natural extracts obtained from the marine halophytes and seaweeds were added to the refined oil (RO) and concentrated methyl esters (CO) at different stages of purification. Accordingly, the EtOAc extracts of *S. brachiata* and *S. maritima* (SBE & SME); the EtOAc fractions from MeOH extracts of *K. alvarezii*, *G. corticata*, *H. musciformis*, *H. valentiae* and *J. rubens* (KAF, GCF, HMF, HVF & JRF) were added to the refined oil at 0.5 % (w/w) and the mixtures of oil plus additives were designated as ROB, ROC, ROD, ROE, ROF, ROG and ROH, respectively. The refined oil without additives was treated as negative control (ROA). The synthetic antioxidants *viz*., α- tocopherol and BHT (0.1%, w/w) were used as positive controls (ROI & ROJ).

Similarly, SBE, SME, KAF, GCF, HMF, HVF and JRF were added to the concentrated fatty acid methyl esters at 0.5 % (w/w) and the mixtures of oil plus additives were designated as COB, COC, COD, COE, COF, COG and COH, respectively. The concentrated fatty acid methyl esters without any additives was treated as negative control (COA). The synthetic antioxidants *viz*., α- tocopherol and BHT (0.1%, w/w) were used as positive controls (COI & COJ, respectively). The P.V., pA..V., TOTOX, TBARS values and DPPH activity (protocols as explained in Chapter 4) were evaluated in an accelerated shelf-life study.

The potential additives that impart stability to the concentrated fatty acid methyl esters were shortlisted by studying their effects in preventing the process of oxidation. Thus three potential additives, *K. alvarezii* (KAF), *H. musciformis* (HMF) and *J. rubens* (JRF) were blended in nine different factorial combinations and added to the concentrated fatty acid methyl esters (CO) at 0.5% (w/w) to obtain 9 treatments (COT_{1-9}) (Table 7.1). The stabilities of these treatments were further evaluated. The degree of synergism (% SYN) was calculated on the basis of the induction time observed as follows (Guzman *et al.*, 2009): $(\%\ SYN) = [IT_{MIX}\text{-}IT_0]\text{-}[(IT_1\text{-}IT_0)+(IT_2\text{-}IT_0)+(IT_3\text{-}IT_0)]*100/[(IT_1\text{-}IT_0)+(IT_2\text{-}\ IT_0)+(IT_3\text{-}IT_0)]$, where IT_{MIX}, IT_0, IT_1, IT_2 and IT_3 are the induction periods of the samples containing the mixture of inhibitors, of the control sample, and of the samples containing the individual

antioxidants. A positive value for percent degree synergism (SYN) defines a synergistic effect between the implicated antioxidants, while a negative value corresponds to an antagonistic effect.

Table 7.1 Different treatments obtained by adding potential additives to the concentrated fatty acid methyl esters (CO)

Treatments	Ratio of potential additives (in g) added to CO
COT_1	KAF + HMF + JRF (0.2:0.2:0.1)
COT_2	KAF + HMF + JRF (0.2:0.1:0.2)
COT_3	KAF + HMF + JRF (0.1:0.2:0.2)
COT_4	KAF + HMF + JRF (0.3:0.1:0.1)
COT_5	KAF + HMF + JRF (0.1:0.3:0.1)
COT_6	KAF + HMF + JRF (0.1:0.1:0.3)
COT_7	KAF + HMF + JRF (0.4:0.5:0.5)
COT_8	KAF + HMF + JRF (0.05:0.4:0.05)
COT_9	KAF + HMF + JRF (0.05:0.05:0.4)

KAF - EtOAc fractions from MeOH extracts of *K. alvarezii*; HMF – EtOAc fractions from MeOH extracts of *H. musciformis* ; JRF - EtOAc fractions from MeOH extracts of *J. rubens*

From the nine combinations of additives (KAF, HMF and JRF) studied, the best combination (COT_3), ie. KAF + HMF + JRF added at the ratio of 0.1:0.2:0.2 (w/w, %) was selected and added to purified sardine fatty acid methyl esters (PO) at different concentrations (0, 0.5, 1 and 2%, w/w) to obtain POA, POB, POC, and POD, respectively and their quality parameters were evaluated. The combination of KAF + HMF + JRF mixed at the ratio of 0.1:0.2:0.2 (COT_3) at 1 % was found to be beneficial for preventing the degradation of purified fatty acid methyl esters (PO).

In order to compare the effect of natural herbal extracts and marine antioxidant combination to the stability of the purified sardine fatty acid methyl esters, the blend of rosemary and green tea (*Chapter 6)* at 1% (w/w) was added to purified fatty acid methyl esters (PO) and designated as POE. A combination of the marine antioxidant blend (KAF + HMF + JRF added at the ratio of 0.1:0.2:0.2) and natural herbal blend at 0.5:0.5 (%) ratio was further added to the PO (named as POF) to study the interactive effect of the combination of marine and natural herbal extract on the degradation of PO, and studied for their oxidative stability. The synthetic additives, α-tocopherol and BHT (at 0.1 % w/w) were used as positive controls (POG and POH). A schematic diagram of the stability study design is represented below (Fig. 7.1.).

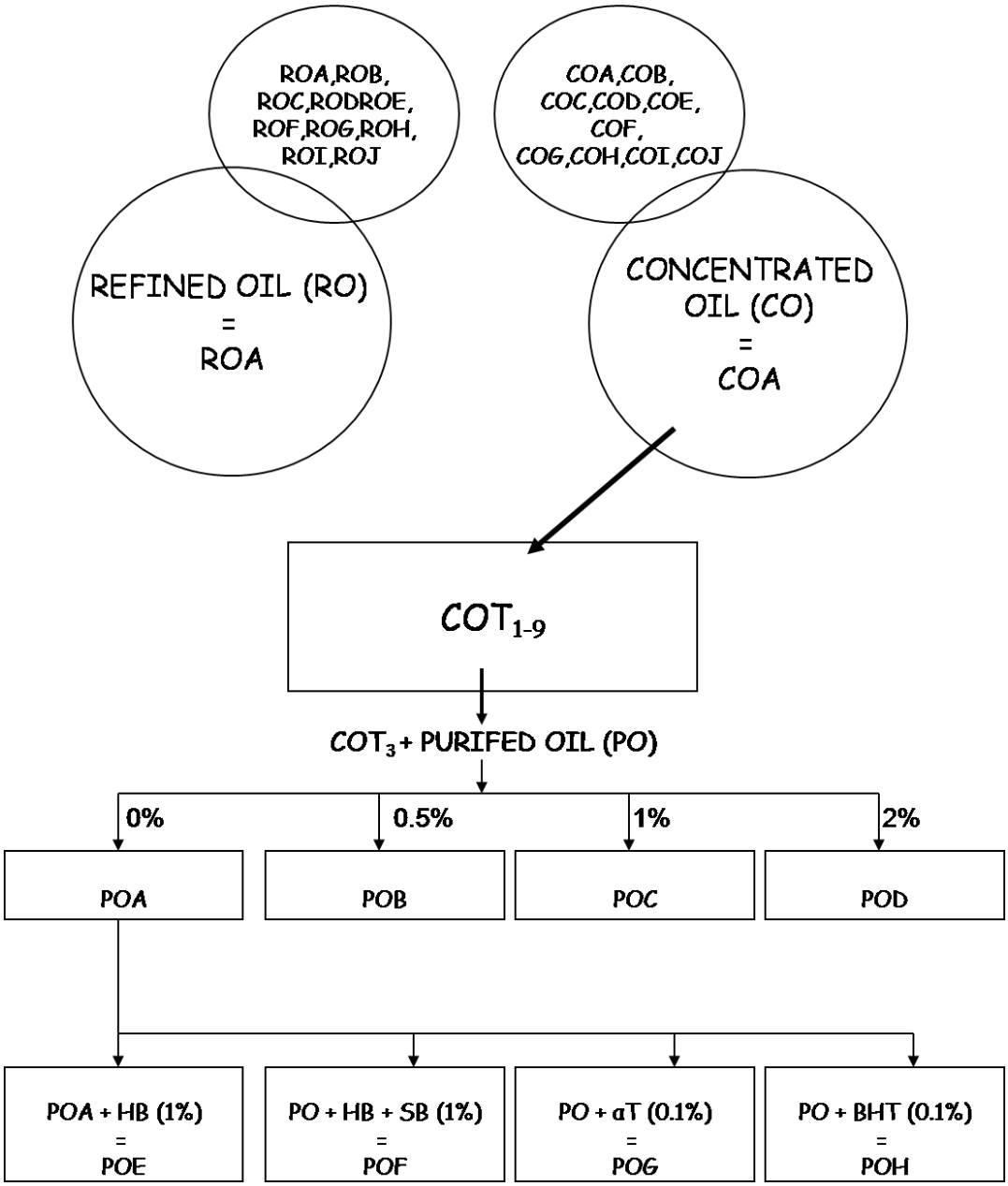


Fig. 7.1. A schematic diagram of the stability study design. The refined oil and concentrated methyl esters (ROA & COA) were added with 0.5% w/w of *S. brachiata* (ROB & COB), *S. maritima* (ROC & COC), *K. alvarezii* (ROD & COD), *G. corticata* (ROE & COE), *H. musciformis* (ROF & COF), *H. valentiae* (ROG & COG) and *J. rubens* (ROH & COH), α-tocopherol and BHT (at 0.1 % w/w; POI & POJ, respectively) and studied for OSI and accelerated stability at 65 °C. The potent additives shortlisted from this preliminary study were added in 9 factorial combinations to the concentrated methyl esters (COT_1- COT_9 as in Table 7.1) and further studied for OSI and accelerated stability at 65 °C. From these combinations, one potent combination was selected (ie. COT_3) and added to purified methyl esters at 0, 0.5, 1 and 2 % w/w concentration to afford POA, POB, POC and POD, respectively and studied for OSI and accelerated stability at 65 °C. The purified methyl esters was further added with herbal blend (1%; POE), herbal blend + COT_3 (0.5 + 0.5%; POF) and compared with α-tocopherol and BHT (at 0.1 % w/w) which used as positive controls (POG and POH) by studying the OSI and accelerated stability at 65 °C.

The additives were added directly to the refined oil and concentrated/purified esters, and stirred in a magnetic stirrer for 15 min at 20-25 ^{o}C.

7.1.3. Assessment of Oxidative Stability of Sardine Oil/Methyl Esters by Accelerated Oxidation Methods

The effect of different additives added to the RO/CO/PO were evaluated using oxidative stability study by using rancimat analysis in 0th day and accelerated stability study using storage trial (using Schaal oven conditions at 65 ^{o}C) at day 0, 4, 8 and 12.

7.1.3.1. Rancimat Analysis

The oxidative stability of oils/methyl esters was determined by measuring their induction period (IT) by rancimat method as described in *Chapter 4*. Analyses were performed in triplicate. The oil/esters in the presence and absence of antioxidants were oxidized under the rancimat conditions and the index of antioxidant activity (AAI) was estimated according to Nwosu et al. (1997) as: AAI = IT with antioxidant/IT without antioxidant.

7.1.3.2. Storage Trial

The specification of the experiments carried out under Schaal oven test conditions (Zandi & Ahmadi, 2000) were as follows. Aliquots of 10 ml of the antioxidant additives in oil solutions and control oil (without additives) were pipetted into 15 ml brown glass vials leaving 10 mm of headspace, flushed with N_2 for 30 s, and then stored in a forced-air oven at 65 ^{o}C (± 1.5 ^{o}C) for 12 days. To estimate the oxidative stability by chemical methods (P.V., pA.V., TOTOX, TBARS (mg MDA equivalent compounds/kg) values, DPPH radical scavenging activity and fatty acid composition) samples were removed periodically at 0, 4, 8 and 12th day from the oven, cooled to room temperature flushed with N_2 for 1 min, before being covered with aluminum foil-parafilm and stored at -20 ^{o}C until analyzed. It is generally accepted that each day (24 h) of storage of oils under Schaal-oven test conditions at 65 ^{o}C is equivalent to one month of storage at ambient temperatures (Evans *et al.,* 1973).

7.1.4. Analysis

7.1.4.1. DPPH Activity of Fish Oil/Esters

The conventional DPPH method uses MeOH as solvent as discussed in Chapter 6. However, to determine the DPPH activity of fish oil, in this chapter, the solvent iso-octane was used for dissolving both DPPH radical and oil samples (Lee *et*

al., 2007). 5 ml of 0.10 mM DPPH in isooctane were mixed with 56 µl oil samples in a 15 ml glass vial and after 30 min standing in the dark; the absorbance of the sample mixture was measured at 517 nm using a UV-VIS-spectrophotometer. Free radical scavenging activity was expressed as % scavenging activity = $(A_0 - As)*100/A_0$, where A_0 = Absorbance of DPPH radical without antioxidant and A_s = Absorbance of DPPH radical with antioxidants.

7.1.4.2. Other Analyses

The protocols for analyzing the fatty acid composition, peroxide value (P.V.), para-anisidine value (pA.V.), total oxidation value (TOTOX) values, TBARS assay, FTIR and ^{1}H NMR analyses performed as detailed in previous chapters (*Chapter 4 & 5*).

7.1.5. Statistical Analysis

All determinations were carried out in triplicate and data is reported as mean ± standard deviation. Significant differences ($p<0.05$) were calculated using Duncan's multiple range test, following a previously reported method.

7.2. Results and Discussion

7.2.1. Stability Study of Refined Oil (RO) and RO Added with Different Additives

7.2.1A. Oil stability Index using Rancimat Analysis

The effects of marine extract additives on the induction times for RO are shown in Table 7.2 (Fig. 7.2). The results showed that the extracts of marine origin (seaweeds and halophytic plants) significantly enhanced the oxidative stability of the refined fish oil. As it has been shown in Table 7.2, the lowest thermal stability in terms of induction time (IT) was for *S. maritima* extract (ROB; 0.25 h). On the other hand, the highest IT was for the red seaweed *H. musciformis* extract (1.26 h), which was higher than the synthetic antioxidant BHT (1.04 h), and superior than that of the control refined oil (ROA; 0.19 h). The antioxidative index (AAI) of the additives exhibited the order: ROF > ROJ > ROD > ROH > ROI > ROG > ROB > ROE > ROC. It is of note that the extracts of *H. musciformis* (ROF), *K. alvarezii* (ROD) *and J. rubens* (ROH) showed higher IT (≥ 0.51 h) than the synthetic antioxidant, α-tocopherol (0.4 h) (Table 7.2). This preliminary experiment using rancimat clearly revealed that the marine natural extracts, especially red seaweed extracts of *H. musciformis* possessed strong protection against the lipid degradation as compared to the control refined oil.

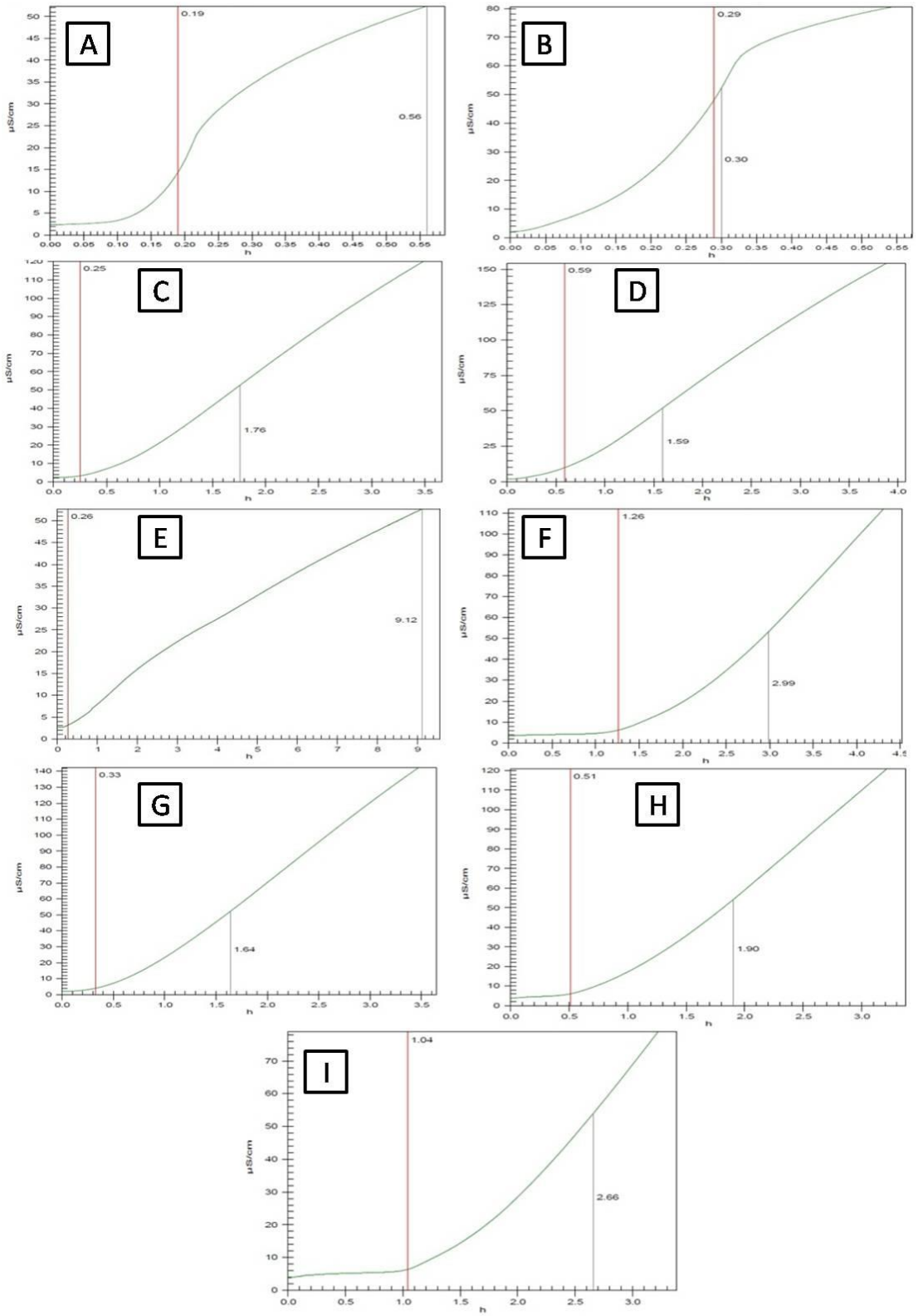


Fig. 7.2. Oil stability indices of (**A**) refined oil ROA and refined oil added with various marine additives (**B**) ROB; (**C**) ROC; (**D**) ROD; (**E**) ROE; (**F**) ROF; (**G**) ROG; (**H**) ROH; (**I**) ROJ

7.2.1B. P.V., pA.V., TOTOX, TBARS Values and DPPH Scavenging Activities during Accelerated Stability Study for 12 days

Primary oxidation products (hydroperoxides) were determined by P.V. measurement. Initially, the difference in peroxide content of control and stabilized oil samples was not noticeable and it became significant ($p<0.05$) after heating upto four days (Table 7.2, Fig. 7.3A-I). In the absence of an antioxidant, the refined oil deteriorated quickly. P.V. increased to 26.3 meqO_2/kg on the 4^{th} day from the initial value of 3.7 meqO_2/kg (at 0^{th} day). However, after 4^{th} day a rapid change in P.V. to was discernable reaching a maximum to 105.2 meqO_2/kg after the 8^{th} day for control oil. Peroxide content for the stabilized samples also increased though the increase was very slow. The P.V. of the refined oil (ROA) increased 31-fold after 12 days to 113.9 meqO_2/kg. With the addition of 0.1 % BHT to the refined oil an increase of P.V. upto 35.8 meqO_2/kg was observed after 12 days. Interestingly, P.V. reached only upto 30.2 meqO_2/kg when *H. musciformis* was added to RO showing the best antioxidant index as compared to the other additives added (d=12). The P.V. results showed that the addition of marine derived antioxidants improved the shelf-life of RO. However, the additives of *S. brachiata*, *S. maritima*, *G. corticata* and *H. valentiae* showed > 11-fold increase in their P.V. values after 12 days of accelerated shelf-life study (d=12). Fish oil, containing high levels of PUFAs, is very susceptible to oxidative deterioration at varying velocities, strongly depending on the storage conditions and fatty acid profile. A peroxide value of 8 meqO_2/kg, an acceptability limit of oil for human consumption, was crossed for all the samples within 4 days (d=4) of accelerated shelf-life study (ie. 4 months at ambient temperature). Earlier study of Boran *et al.*, (2006) reported that during storage at 4°C, the peroxide values of several fish oils reached the acceptable limit of 8 meqO_2/kg after 60 or 90 days and after at least 150 days at –18°C. This fact corroborates well with the present work that the storage temperature had significant effects on the storage stability of the fish oil. The P.V. results apparently indicated that refined oil supplemented with marine antioxidant extracts is stable for 4 months when stored at the ambient temperature.

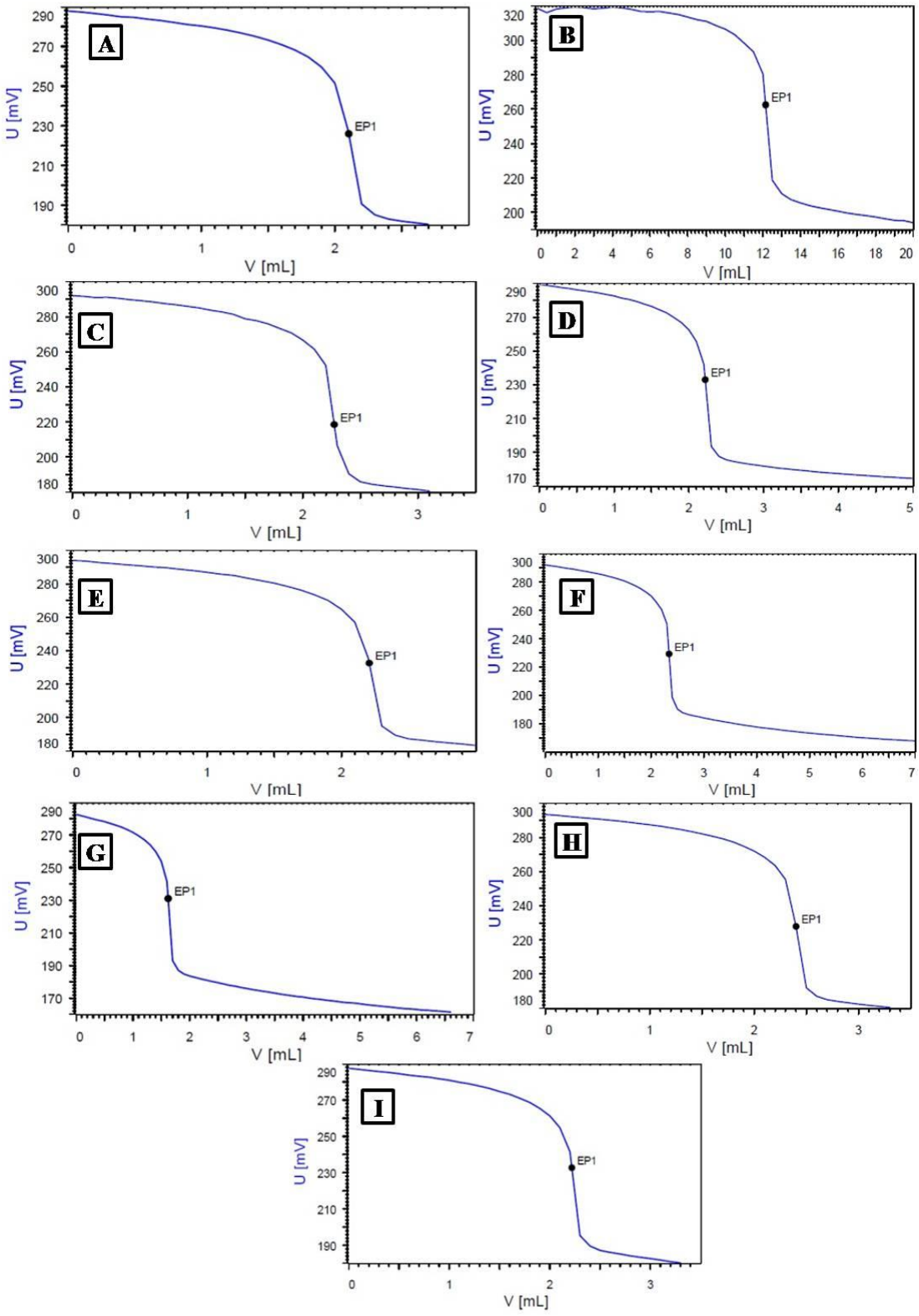


Fig. 7.3. Potentiometric titration curves obtained using an automatic titrator indicating the peroxide levels in (**A**) refined oil ROA at 12$^{th\ day}$ of accelerated stability study (**B**) ROB; (**C**) ROC; (**D**) ROD; (**E**) ROE; (**F**) ROF; (**G**) ROG (**H**) ROH; (**I**) ROJ

Formation of the secondary oxidation products was expressed by an increase in the para-anisidine values. The pA.V. was found to increase 6.5-fold for ROA, and only up to 3, 2.8 and 2.4-fold for ROD, ROF and ROH, respectively after 12 days (d = 12). However, the synthetic antioxidants, α-tocopherol (ROI) and BHT (ROJ) showed 3 & 3.2 -fold increase, respectively in their pA.V. after 12 days of accelerated shelf-life studies (Table 7.2).

The TOTOX value for the control refined oil (ROA) showed 17.7 -fold increment after 12 days. The treatments ROD, ROF and ROH exhibited TOTOX value increment in the order, ROD > ROH > ROF (6.8, 5.9 & 5.4 -fold, respectively) showing the beneficiary effect of the purified extracts obtained from *H. musciformis*, *J. rubens* and *K. alvarezii*, were added to the refined oil (Table 7.2).

The TBARS value is an index of lipid oxidation measuring malonaldehyde (MDA) content. MDA is formed through hydroperoxides, which is the initial reaction product of lipid oxidation. The oxidation of unsaturated fatty acids containing 3 or more double bonds has been shown to contribute to the formation of TBARS (Rael *et al*., 2004). No significant differences in TBARS concentration among the treatments were observed during the storage period, while the control oil (ROA) showed significant change in its TBARS inhibitory activity with an increase upto 7.3 MDAEQ/kg from 3.7 MDAEQ/kg (Table 7.2). However, the TBARS values increased progressively over the entire storage period. The general increase in TBARS values during the storage period was due to the fact that, as oxidation proceeds, lipid hydroperoxides break down to produce the secondary oxidation products, as supported by the findings of Strange et al. (1997). The lower TBARS values observed for ROB - ROJ indicated that the lipid oxidation did not progress significantly during the accelerated storage for the RO supplemented with additives (ROB - ROH). Correspondingly, a significant increase in TBARS values for ROA indicated the loss of the natural antioxidants in the refined fish oil ($p<0.05$). While comparing the TBARS values among the samples, the control sample contained the

higher TBARS value than the other samples during the 12 days of accelerated storage ($p<0.05$). During the first 8 days of storage, the samples added with the halophyte extracts (ROB & ROC) had the highest TBARS values as compared with those supplemented with other additives ($p<0.05$). In general, no significant difference in TBARS values were obtained among the samples added with any additives ($p<0.05$).

The DPPH activity of the control oil reduced to 16.3 % from 76.3% (a percent reduction of 78.6 %) after 12 days of accelerated stability study (Table 7.2). The results of this study showed that refined oils containing free radical scavenging compounds as in the various additives can increase the DPPH scavenging activity. For RO without any additives (ROA), the generated free radicals reacted with the DPPH and an increase in activity from the initial value (d=0) was recorded in the 4^{th} day (78.1 %). However, in case of samples with the antioxidant additives, free radicals from oxidized refined fish oil reacted firstly with the additives, whereas the remaining additives might be reacted with the DPPH free radical to stabilize the latter, thereby showing better free radical scavenging activity than the control refined oil.

In general, the P.V., pA.V., TBARS values of the samples were gradually increased, during incubation at 65 ^{o}C, whereas the increment was found to be slower for the refined oil supplemented with antioxidant additives.

Table 7.2 Characteristics of refined oil (ROA), refined oil added with various marine extracts (ROB - ROH) and synthetic antioxidants (ROI & ROJ) during accelerated stability study at 65 °C for 12 days.

Parameters	Days	ROA	ROB	ROC	ROD	ROE	ROF	ROG	ROH	ROI	ROJ
IT		0.19	0.29	0.25	0.59	0.26	1.26	0.33	0.51	0.4	1.04
ST		0.56	0.3	1.76	1.59	9.12	2.99	1.64	1.9	1.02	2.66
AAI			1.53	1.32	3.11	1.37	6.63	1.74	2.68	2.11	5.47
P.V.	0	3.68±0.07^{a}	3.65±0.37^{a}	3.89±0.39^{a}	4.01±0.4^{a}	3.68±0.37^{a}	3.69±0.37^{a}	3.45±0.35^{a}	3.78±0.08^{a}	3.84±0.38^{a}	3.77±0.38^{a}
	4	26.31±0.63^{b}	18.29±0.83^{b}	22.97±0.3^{b}	21.16±0.12^{b}	20.87±0.09^{b}	13.16±0.32^{b}	16.36±0.64^{b}	14.02±0.4^{b}	14.11±0.41^{b}	12.54±0.25^{b}
	8	105.23±0.52^{c}	34.44±0.44^{c}	36.31±0.63^{c}	33.13±0.31^{c}	37.21±0.72^{c}	28.54±0.85^{c}	37.46±0.75^{c}	31.21±0.12^{c}	32.55±0.26^{c}	26.55±0.66^{c}
	12	113.86±0.39^{c}	43.9±0.39^{d}	43.59±0.36^{d}	40.88±0.09^{c}	42.74±0.27^{c}	30.24±0.02^{c}	41.33±0.13^{c}	40.15±0.02^{c}	40.13±0.01^{c}	35.75±0.58^{d}
pA.V.	0	8.68±0.07^{a}	9.1±0.91^{a}	8.26±0.83^{a}	7.34±0.73^{a}	8.02±0.8^{a}	7.96±0.8^{a}	8.46±0.85^{a}	9.99±0.01^{a}	9.1±0.91^{a}	9.26±0.93^{a}
	4	22.6±0.26^{b}	18.9±0.89^{b}	18.2±0.82^{b}	14.36±0.44^{b}	17.54±0.75^{b}	15.34±0.53^{b}	18.14±0.81^{b}	17.01±0.7^{b}	14.23±0.42^{a}	12.21±0.22^{a}
	8	40.4±0.04^{c}	39.85±0.99^{c}	38.25±0.83^{c}	19.94±0.99^{c}	25.46±0.55^{c}	21.36±0.14^{c}	21.36±0.14^{c}	22.36±0.24^{c}	22.1±0.21^{b}	22.16±0.22^{b}
	12	56.2±0.62^{d}	44.51±0.45^{d}	49.65±0.97^{d}	22.36±0.24^{c}	29.27±0.93^{c}	22.04±0.2^{c}	24.12±0.41^{c}	24.12±0.41^{c}	26.86±0.69^{b}	29.34±0.93^{c}
TOTOX	0	16.04±0.6^{a}	16.4±0.64^{a}	16.04±0.6^{a}	15.36±0.54^{a}	15.38±0.54^{a}	15.34±0.53^{a}	15.36±0.54^{a}	17.55±0.76^{a}	16.78±0.68^{a}	16.8±0.68^{a}
	4	75.22±0.52^{b}	55.48±0.55^{b}	64.14±0.41^{b}	56.68±0.67^{b}	59.28±0.93^{b}	41.66±0.17^{b}	50.86±0.09^{b}	45.05±0.51^{b}	42.45±0.25^{b}	37.29±0.73^{b}
	8	250.86±0.09^{c}	108.73±0.87^{c}	110.87±0.09^{c}	86.2±0.62^{c}	99.88±0.99^{c}	78.44±0.84^{c}	96.28±0.63^{c}	84.78±0.48^{c}	87.2±0.72^{c}	75.26±0.53^{c}
	12	283.92±0.39^{d}	132.31±0.23^{d}	136.83±0.68^{d}	104.12±0.41^{d}	114.75±0.48^{d}	82.52±0.25^{c}	106.78±0.68^{d}	104.42±0.44^{d}	107.12±0.71^{d}	100.84±0.08^{d}
TBARS	0	3.69±0.37^{a}	3.61±0.36^{a}	3.63±0.36^{a}	3.41±0.34^{a}	3.54±0.35^{a}	3.43±0.34^{a}	3.48±0.35^{a}	3.58±0.36^{a}	3.32±0.33^{a}	3.28±0.33^{a}
	4	3.86±0.39^{a}	3.72±0.37^{a}	3.68±0.37^{a}	3.53±0.35^{a}	3.63±0.36^{a}	3.45±0.35^{a}	3.56±0.36^{a}	3.69±0.07^{a}	3.36±0.34^{a}	3.31±0.33^{a}
	8	3.81±0.38^{a}	3.79±0.38^{a}	3.79±0.38^{a}	3.58±0.36^{a}	3.76±0.38^{a}	3.59±0.36^{a}	3.65±0.37^{a}	3.71±0.37^{a}	3.41±0.34^{a}	3.36±0.34^{a}
	12	7.27±0.73^{b}	4.36±0.44^{a}	3.92±0.39^{a}	3.71±0.37^{a}	3.84±0.38^{a}	3.64±0.36^{a}	3.88±0.39^{a}	3.75±0.38^{a}	3.58±0.36^{a}	3.48±0.35^{a}
DPPH	0	76.27±0.63^{a}	79.26±0.93^{a}	80.36±0.04^{a}	92.36±0.24^{a}	84.12±0.41^{a}	95.63±0.56^{a}	85.27±0.53^{a}	96.27±0.63^{a}	95.63±0.56^{a}	98.28±0.83^{a}
	4	78.14±0.81^{a}	70.31±0.03^{b}	71.31±0.13^{b}	88.31±0.83^{a}	80.11±0.01^{a}	90.91±0.09^{a}	76.21±0.62^{a}	90.31±0.03^{a}	91.34±0.13^{a}	92.26±0.23^{a}
	8	45.68±0.07^{b}	68.34±0.83^{b}	67.26±0.73^{b}	87.64±0.76^{a}	79.12±0.91^{a}	76.64±0.66^{b}	61.31±0.13^{b}	84.32±0.43^{b}	81.36±0.14^{b}	81.36±0.14^{b}
	12	16.31±0.03^{c}	46.21±0.62^{c}	37.31±0.73^{c}	69.26±0.93^{b}	54.3±0.43^{b}	71.31±0.13^{b}	50.48±0.05^{c}	70.46±0.05^{c}	68.39±0.84^{c}	74.45±0.45^{c}

Data are expressed as mean ± standard deviation of three replicates. Means with different superscripts (a, b, etc) in the column indicates a statistical difference ($p<0.05$).ROA = RO - Refined oil (Chapter 4) without any additives (Control); ROB – RO + *S. brachiata* extract (0.5%), ROC – RO + *S. maritima* extract (0.5%), ROD – RO + *K. alvarezii* extract (0.5%), ROE – RO + *G. corticata* extract (0.5%), ROF – RO + *H. musciformis* extract (0.5%), ROG - RO + *H. valentiae* extract (0.5%), ROH – RO + *J. rubens* extract (0.5%), ROI – RO + α-tocopherol (0.1%) and ROJ - RO + BHT (0.1%); IT – Induction time in h; ST – Stability time in h; AAI - Antioxidant activity index = IT with antioxidant/IT without antioxidant; P.V. – Peroxide value represented in meqO_2/kg; pA.V. – p-anisidine value; TOTOX – total oxidation value (2 x P.V. + pA.V.); TBARS – Thiobarbituric acid reactive species represented in mg MDA equivalent compounds/kg sample (MDAEQ/kg); DPPH – free radical scavenging activity (%).

7.2.1C. Fatty Acid Composition during Accelerated Stability Study for 12 Days

A significant reduction in EPA and DHA was observed over the storage trial in the refined control oil (ROA) as the LC-PUFA are more susceptible to oxidation than other fatty acids, such as saturated or monounsaturated (Table 7.3A-D). A decrease in the amount of *n*-3 PUFAs over the storage trial is an indication of oxidation of the oil. *H. musciformis*, *J. rubens* and *K. alvarezii* extracts showed a marked level of protection towards the essential PUFAs. The percent reduction of EPA after 12th day was higher for ROA (56.2 %), followed by those of ROG (51.6 %), ROE (45.2%), ROC (42.4 %), ROB (13.9 %), ROI (20.3 %), ROD (18.9 %), ROH (15.1 %), ROF (12.8 %) and ROJ (6 %) (Fig. 7.4). The percent reduction in DHA content after 12 days of accelerated shelf life study was in the order, ROA (47.1 %) > ROC (33.7 %) > ROB (31.9 %) > ROE (30.8 %) > ROD (27.1 %) > ROI (26%) > ROG (24.7 %) > ROF (20.4 %) > ROH (12.7 %) > ROJ (7.4%). This indicated that ROD, ROH and ROF could able to prevent the oxidation of fatty acids of the refined oil better than the synthetic antioxidant, α-tocopherol. The refined oil containing *S. maritima* and *S. brachiata* extracts showed similar patterns in *n*-3 PUFA content to that of refined oil used as control over the storage trial, suggesting that these extracts were not effective to prevent the oxidation of PUFAs.

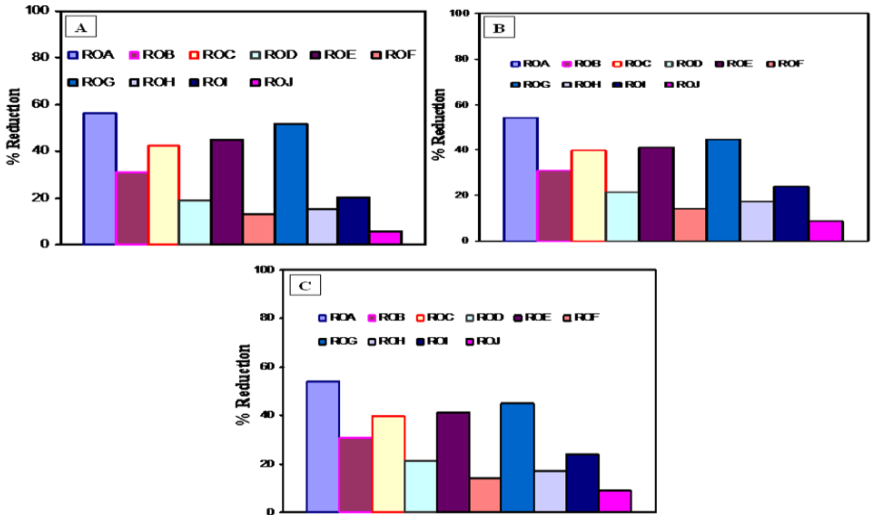


Fig. 7.4. Percent reduction in (**A**) EPA, (**B**) DHA and (**C**) ∑*n*-3PUFA after 12 days of accelerated shell life study of refined oil ROA, oil added with various additives, ROB-ROH and synthetic additives (ROI & ROJ) compared to the 0th day

Table 7.3A Fatty acid composition of ROA, ROB and ROC during accelerated storage for 12 days

	ROA				ROB				ROC			
Days	0	4	8	12	0	4	8	12	0	4	8	12
Fatty acids Saturated												
14:0	12.91±0.29	11.26±0.13	12.25±0.23	14±0.04	12.64±0.26	12.69±0.27	13.88±0.39	14.01±0.04	12.85±0.29	13.01±0.03	13.22±0.32	14.44±0.44
15:0	0.68±0.07	0.84±0.08	0.99±0.1	1.11±0.11	0.83±0.08	0.91±0.09	1.01±0.1	0.89±0.09	1.1±0.11	0.98±0.1	1.02±0.1	0.96±0.1
16:0	21.8±0.18	19.61±0.96	21.44±0.14	24.14±0.41	18.63±0.86	21.24±0.12	22.35±0.24	23.65±0.37	19.23±0.92	22.15±0.22	22.63±0.26	26.32±0.63*
17:0	0.73±0.07	0.95±0.1	1.02±0.1	1.21±0.12	1.05±0.11	0.91±0.09	1.02±0.1	1.12±0.11	1.05±0.11	1.12±0.11	1.25±0.13	1.25±0.13
18:0	4.31±0.41	5.72±0.55	5.78±0.57	5.87±0.6	4.5±0.44	4.65±0.45	5.21±0.5	5.32±0.51	5.23±0.52	4.69±0.46	4.66±0.46	4.77±0.48
20:0	0.34±0.03	1.01±0.1	1.11±0.11	1.14±0.11	0.32±0.03	1.01±0.1	1.11±0.11	1.12±0.11	0.88±0.09	0.99±0.1	0.99±0.1	0.99±0.1
22:0	0.05±0.01	0.43±0.04	0.33±0.03	0.07±0.01	0.11±0.01	0.15±0.02	0.18±0.02	0.13±0.01	0.39±0.04	0.13±0.01	0.14±0.01	0.16±0.02
24:0	0.03±0	0.06±0.01	0.04±0	0.05±0.01	0.06±0.01	0.09±0.01	0.1±0.01	0.11±0.01	0.07±0.01	0.09±0.01	0.1±0.01	0.21±0.02
∑SFA	40.86±0.09	39.88±0.09	42.96±0.03	47.59±0.76	38.14±0.81	41.65±0.17	44.86±0.49	46.35±0.64	40.8±0.08	43.16±0.32	44.01±0.04	49.1±0.91
Monounsaturated												
14:1*n*-7	0.13±0.01	0.08±0.01	0.09±0.01	0.11±0.01	0.08±0.01	0.09±0.01	0.11±0.01	0.11±0.01	0.11±0.01	0.12±0.01	0.12±0.01	0.12±0.01
15:1*n*-7	0.16±0.02	0.02±0	0.02±0	0.3±0.03	0.02±0	0.22±0.02	0.22±0.02	0.22±0.02	0.02±0	0.22±0.02	0.23±0.02	0.23±0.02
16:1*n*-7 *trans*	0.04±0	0.01±0	0.01±0	ND	0.01±0	ND	ND	ND	ND	ND	ND	ND
16:1*n*-7	13.22±0.32	12.06±0.21	13.02±0.3	14.98±0.05	13.63±0.36	13.65±0.37	13.98±0.04	14.01±0.4	14.02±0.4	13.25±0.33	13.36±0.34	14.32±0.43
18:1*n*-9 *trans*	0.03±0	ND	ND	ND	0.08±0.01	ND	ND	ND	ND	ND	ND	ND
18:1*n*-9	9.53±0.95	10.24±0.02	10.36±0.04	11.74±0.17	12.82±0.28	11.25±0.13	11.56±0.16	12.23±0.22	10.58±0.06	11.22±0.12	11.36±0.14	12.22±0.22
20:1*n*-9	0.6±0.06	0.72±0.07	0.75±0.08	0.73±0.07	0.8±0.08	0.76±0.08	0.88±0.09	0.98±0.1	0.73±0.07	0.91±0.09	1.02±0.1	1.02±0.1
22:1*n*-9	2.94±0.29	2.17±0.22	1.69±0.17	1.52±0.15	2.43±0.24	2.25±0.23	2.69±0.27	2.65±0.27	2.69±0.27	1.32±0.13	1.33±0.13	1.33±0.13
24:1*n*-9	0.16±0.02	0.32±0.03	0.44±0.04	0.51±0.05	0.84±0.08	0.68±0.07	0.69±0.07	0.71±0.07	0.98±0.1	0.65±0.07	0.97±0.1	0.97±0.1
∑MUFA	26.79±0.68	25.62±0.56	26.38±0.64	29.89±0.99	30.71±0.07	28.9±0.89	30.13±0.01	30.91±0.09	29.13±0.91	27.69±0.77	28.39±0.84	30.21±0.02
Polyunsaturated												
18:2*n*6 *trans*	0.03±0	0.01±0	0.01±0	0.01±0	0.09±0.01	0.02±0	0.02±0	0.01±0	ND	0.03±0	0.06±0.01	0.05±0.01
18:2*n*-6	1.89±0.19	2.11±0.21	2.22±0.22	2.28±0.23	2.28±0.23	2.02±0.2	2.02±0.2	2.01±0.2	2.01±0.2	1.84±0.18	1.36±0.14	1.22±0.12
18:3*n*-6	0.41±0.04	0.47±0.05	0.48±0.05	0.49±0.05	0.46±0.05	0.56±0.06	0.53±0.05	0.52±0.05	0.54±0.05	0.66±0.07	0.74±0.07	0.78±0.08
18:3*n*-3	0.66±0.07	0.05±0.01	0.07±0.01	0.13±0.01	0.14±0.01	0.08±0.01	0.06±0.01	0.05±0.01	ND	0.04±0	0.06±0.01	0.11±0.01
20:2*n*-6	1.25±0.13	2.15±0.22	1.78±0.18	1.65±0.17	2.59±0.26	2.12±0.21	1.95±0.2	1.89±0.19	2.68±0.27	2.12±0.21	1.55±0.16	1.22±0.12
20:3*n*-6	0.13±0.01	0.17±0.02	0.22±0.02	0.34±0.03	0.12±0.01	0.14±0.01	0.13±0.01	0.11±0.01	0.39±0.04	0.16±0.02	0.19±0.02	0.21±0.02
20:4*n*-6	0.49±0.05	0.63±0.06	0.56±0.06	0.55±0.06	0.74±0.07	0.55±0.06	0.52±0.05	0.52±0.05	0.89±0.09	0.52±0.05	0.71±0.07	0.55±0.06
20:5*n*-3 EPA	14.67±0.47	12.43±0.24	9.56±0.96*	6.42±0.64*	14.46±0.45	13.02±0.3	10.32±0.03	9.99±0.01	15.02±0.05	13.69±0.37	12.01±0.02	8.65±0.87*
22:5*n*-3	0.53±0.05	1.23±0.12	1.23±0.12	0.33±0.03	0.79±0.08	0.7±0.07	0.69±0.07	0.65±0.07	0.78±0.08	0.73±0.07	0.76±0.08	0.48±0.05
22:6*n*-3 DHA	5.71±0.57	5.53±0.55	4.65±0.47	3.02±0.3	4.89±0.49	4.02±0.4	3.98±0.4	3.33±0.33	5.01±0.5	4.01±0.4	3.81±0.38	3.32±0.33
∑PUFA	25.77±0.58	24.78±0.48	20.78±0.08*	15.22±0.52*	26.56±0.66	23.23±0.32	20.22±0.02	19.08±0.91	27.32±0.73	23.8±0.38	21.25±0.13	16.59±0.66
EPA+DHA	20.38±0.04	17.96±0.8	14.21±0.42*	9.44±0.94*	19.35±0.94	17.04±0.7	14.3±0.43	13.32±0.33	20.03±0.02	17.7±0.77	15.82±0.58	11.97±0.02
∑*n*-3PUFA	21.57±0.16	19.24±0.92	15.51±0.55*	9.9±0.99*	20.28±0.03	17.82±0.78	15.05±0.51	14.02±0.4	20.81±0.08	18.47±0.85	16.64±0.66	12.56±0.26
∑n-6PUFA	4.17±0.42	5.53±0.55	5.26±0.53	5.31±0.53	6.19±0.62	5.39±0.54	5.15±0.52	5.05±0.51	6.51±0.65	5.3±0.53	4.55±0.46	3.98±0.4
∑*n*-3/∑*n*-6	5.17±0.52	3.48±0.35	2.95±0.3	1.86±0.19*	3.28±0.33	3.31±0.33	2.92±0.29	2.78±0.28	3.2±0.32	3.48±0.35	3.66±0.37	3.16±0.32
∑*n*-6/∑*n*-3	0.19±0.02	0.29±0.03	0.34±0.03	0.54±0.05	0.31±0.03	0.3±0.03	0.34±0.03	0.36±0.04	0.31±0.03	0.29±0.03	0.27±0.03	0.32±0.03
∑PUFA/∑SFA	0.63±0.06	0.62±0.06	0.48±0.05	0.32±0.03	0.7±0.07	0.56±0.06	0.45±0.05	0.41±0.04	0.67±0.07	0.55±0.06	0.48±0.05	0.34±0.03
∑TRANS	0.1±0.01	0.02±0	0.02±0	0.01±0	0.18±0.02	0.02±0	0.02±0	0.01±0	ND	0.03±0	0.06±0.01	0.05±0.01

ressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total ted fatty acids. Means with different superscripts (a, b, c, d) in the same row indicates a statistical difference ($p<0.05$). ND: not detected. ROA - Refined pter 4) without any additives (Control); ROB – RO + *S. brachiata* extract (0.5%), ROC – RO + *S. maritima* extract (0.5%).

Table 7.3B Fatty acid composition of ROD, ROE and ROF during accelerated storage for 12 days

	ROD				ROE				ROF			
Days	0	4	8	12	0	4	8	12	0	4	8	12
Fatty acidsSaturated												
14:0	10.84±0.08	11.01±0.1	13.07±0.31	11.97±0.2	13.67±0.37	12.55±0.26	13.26±0.33	15.01±0.5	12.06±0.21	12.07±0.21	12±0.02	11.8±0.18
15:0	0.92±0.09	0.92±0.09	0.95±0.1	0.86±0.09	0.94±0.09	0.98±0.1	1±0.1	0.98±0.1	0.9±0.09	0.91±0.09	0.9±0.09	0.86±0.09
16:0	18.65±0.87	18.65±0.87	25.01±0.5	20.84±0.08	18.69±0.85	22.01±0.01	26.02±0.6	27.01±0.08	21.86±0.17	21.9±0.19	21.82±0.18	21.63±0.19
17:0	1.14±0.11	1.15±0.12	1.09±0.11	0.98±0.1	1.04±0.1	1.11±0.11	1.11±0.11	1.32±0.13	1.06±0.11	1.06±0.11	1.07±0.11	1.09±0.11
18:0	4.63±0.46	4.6±0.47	5.24±0.51	4.59±0.46	4.63±0.44	5.26±0.54	5.22±0.52	5.35±0.54	4.69±0.47	4.7±0.47	4.81±0.48	4.89±0.49
20:0	0.33±0.03	0.25±0.03	1.02±0.1	0.93±0.09	0.31±0.03	0.95±0.1	0.98±0.1	1.01±0.1	0.97±0.1	0.98±0.1	1.01±0.1	1.03±0.1
22:0	0.1±0.01	0.12±0.01	0.64±0.06	0.1±0.01	0.1±0.01	0.44±0.04	0.45±0.05	0.65±0.07	0.12±0.01	0.13±0.01	0.3±0.03	0.33±0.03
24:0	0.05±0.01	0.04±0	0.11±0.01	0.05±0.01	0.07±0.01	0.09±0.01	0.11±0.01	0.12±0.01	0.08±0.01	0.07±0.01	0.06±0.01	0.06±0.01
∑SFA	36.66±0.67	36.74±0.67	47.13±0.71	40.32±0.03	39.45±0.95	43.39±0.34	48.15±0.82	51.45±0.15	41.74±0.17	41.82±0.18	41.97±0.02	41.69±0.17
Monounsaturated												
14:1*n*-7	0.09±0.01	0.09±0.01	0.1±0.01	0.1±0.01	0.09±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.09±0.01	0.09±0.01	0.08±0.01	0.08±0.01
15:1*n*-7	0.02±0	0.01±0	0.03±0	0.16±0.02	0.02±0	0.26±0.03	0.21±0.02	0.21±0.02	0.02±0	0.02±0	0.13±0.01	0.16±0.02
16:1*n*-7 *trans*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
16:1*n*-7	14.24±0.42	14.69±0.47	12.86±0.29	11.83±0.18	14.24±0.41	12.65±0.28	12.65±0.27	13.55±.36	12.28±0.23	12.36±0.24	12.04±0.02	11.97±0.01
18:1*n*-9 *trans*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
18:1*n*-9	10.07±0.01	10.11±0.01	10.93±0.09	10.33±0.03	12.16±0.22	8.01±0.8	7.89±0.79	7.54±0.75	12.29±0.23	11.31±0.13	10.21±0.02	10.21±0.02
20:1*n*-9	0.79±0.08	0.79±0.08	0.65±0.07	0.76±0.08	0.79±0.08	0.65±0.07	0.65±0.07	0.69±0.07	0.74±0.07	0.75±0.08	0.7±0.07	0.66±0.07
22:1*n*-9	2.56±0.26	2.65±0.27	2.2±0.22	2.13±0.21	2.56±0.25	2.01±0.2	2.22±0.22	2.22±0.22	2.31±0.23	2.33±0.23	2.01±0.2	2±0.2
24:1*n*-9	0.36±0.04	0.62±0.06	0.24±0.02	0.26±0.03	1.36±0.14	0.97±0.1	0.97±0.1	0.99±0.1	0.29±0.03	0.31±0.03	0.46±0.05	0.68±0.07
∑MUFA	28.13±0.81	28.96±0.9	27.01±0.07	25.57±0.56	31.22±0.12	24.65±0.47	24.69±0.47	25.3±0.53	28.02±0.8	27.17±0.72	25.63±0.56	25.76±0.58
Polyunsaturated												
18:2*n*6 *trans*	0.01±0	0.01±0	0.01±0	0.01±0	0.09±0.01	0.02±0	ND	0.02±0	0.01±0	0.01±0	0.01±0	ND
18:2*n*-6	2.52±0.25	2.34±0.23	1.99±0.2	2.16±0.22	2.57±0.26	2.25±0.23	2.11±0.21	2.01±0.2	2.1±0.21	2.1±0.21	±0	1.95±0.2
18:3*n*-6	0.44±0.04	0.4±0.04	0.41±0.04	0.46±0.05	0.66±0.07	0.66±0.07	0.55±0.06	0.56±0.06	0.46±0.05	0.45±0.05	0.41±0.04	0.42±0.04
18:3*n*-3	0.42±0.04	0.42±0.04	0.14±0.01	0.11±0.01	0.46±0.05	0.21±0.02	0.21±0.02	0.11±0.01	0.12±0.01	0.13±0.01	0.14±0.01	0.12±0.01
20:2*n*-6	2.93±0.29	2.88±0.29	1.97±0.2	2.19±0.22	3.35±0.33	2.85±0.28	2.74±0.27	2.88±0.29	2.25±0.23	2.23±0.22	2.13±0.21	2.01±0.2
20:3*n*-6	0.08±0.01	0.08±0.01	0.09±0.01	0.16±0.02	0.09±0.01	0.14±0.01	0.11±0.01	0.14±0.01	0.2±0.02	0.16±0.02	0.17±0.02	0.16±0.02
20:4*n*-6	0.84±0.08	0.81±0.08	0.17±0.02	0.57±0.06	1.21±0.12	1.02±0.1	1.01±0.1	0.98±0.1	0.63±0.06	0.54±0.05	0.59±0.06	0.68±0.07
20:5*n*-3 EPA	15.46±0.55	13.65±0.37	14.11±0.41	12.53±0.25	14.63±0.46	12.01±0.02	10.25±0.03	8.02±0.8 *	14.16±0.42	13.01±0.3	12.83±0.28	12.34±0.23
22:5*n*-3	0.67±0.07	0.71±0.07	0.87±0.09	0.65±0.07	0.79±0.08	0.7±0.07	0.56±0.06	0.74±0.07	1.56±0.16	1.56±0.16	1.5±0.15	1.45±0.15
22:6*n*-3 DHA	5.2±0.52	4.84±0.48	4.27±0.43	3.79±0.38	4.86±0.49	4.85±0.49	3.89±0.39	3.36±0.34	4.99±0.5	4.01±0.4	3.96±0.4	3.97±0.4
∑PUFA	28.57±0.86	26.14±0.61	24.03±0.04	22.63±0.26	28.71±0.87	24.71±0.47	21.43±0.14	18.82±0.88	26.48±0.65	24.2±0.42	21.74±0.17	23.1±0.31
EPA+DHA	20.66±0.07	18.49±0.85	18.38±0.84	16.32±0.63	19.49±0.95	16.86±0.69	14.14±0.41	11.38±0.14	19.15±0.92	17.02±0.07	16.79±0.68	16.31±0.63
∑*n*-3PUFA	21.75±0.18	19.62±0.96	19.39±0.94	17.08±0.71	20.74±0.07	17.77±0.78	14.91±0.49	12.23±0.22	20.83±0.08	18.71±0.87	18.43±0.84	17.88±0.79
∑*n*-6PUFA	6.81±0.68	6.51±0.65	4.63±0.46	5.54±0.55	7.88±0.79	6.92±0.69	6.52±0.65	6.57±0.66	5.64±0.56	5.48±0.55	3.3±0.33	5.22±0.52
∑*n*-3/∑*n*-6	3.19±0.32	3.01±0.3	4.19±0.42	3.08±0.31	2.63±0.26	2.57±0.26	2.29±0.23	1.86±0.19	3.69±0.37	3.41±0.34	5.58±0.56	3.43±0.34
∑*n*-6/∑*n*-3	0.31±0.03	0.33±0.03	0.24±0.02	0.32±0.03	0.38±0.04	0.39±0.04	0.44±0.04	0.54±0.05	0.27±0.03	0.29±0.03	0.18±0.02	0.29±0.03
∑PUFA/∑SFA	0.78±0.08	0.71±0.07	0.51±0.05	0.56±0.06	0.73±0.07	0.57±0.06	0.45±0.05	0.37±0.04	0.63±0.06	0.58±0.06	0.52±0.05	0.55±0.06
∑TRANS	0.01±0	0.01±0	0.01±0	0.01±0	0.09±0.01	0.02±0	ND	0.02±0	0.01±0	0.01±0	0.01±0	ND

essed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. Means with scripts (a, b, c, d) in the same row indicates a statistical difference ($p<0.05$). ND: not detected. ROD – RO + *K. alvarezii* extract (0.5%), ROE – RO + *G. corticata* extract (0.5%), ROF – RO + *H.* xtract (0.5%).

Table 7.3C Fatty acid composition of ROG and ROH during accelerated storage for 12 days

	ROG				ROH			
Days	**0**	**4**	**8**	**12**	**0**	**4**	**8**	**12**
Fatty acids Saturated								
14:0	10.84±0.08	12.44±0.24	14.05±0.41	12.14±0.21	9.56±0.96	13.37±0.34	13.61±0.36	13.71±0.37
15:0	0.95±0.1	0.98±0.1	1.01±0.1	0.98±0.1	0.91±0.09	0.96±0.1	0.96±0.1	0.97±0.1
16:0	19.11±0.9	22.35±0.23	28.04±0.8	28.01±0.8	19.01±0.09	23.68±0.37	23.46±0.35	24.56±0.46
17:0	1.25±0.13	1.11±0.11	1.11±0.11	1.33±0.13	1.12±0.11	1.1±0.11	1.04±0.1	1.14±0.11
18:0	4.53±0.45	5.26±0.53	5.22±0.52	7.36±0.74	4.74±0.47	5.07±0.51	5.11±0.51	5.15±0.52
20:0	0.33±0.03	0.95±0.1	1.01±0.01	1.2±0.12	0.34±0.03	1±0.1	0.68±0.07	0.63±0.06
22:0	0.14±0.01	0.44±0.04	0.45±0.05	0.65±0.07	0.11±0.01	0.2±0.02	0.36±0.04	0.43±0.04
24:0	0.08±0.01	0.09±0.01	0.15±0.02	0.13±0.01	0.04±0	0.1±0.01	0.16±0.02	0.19±0.02
∑SFA	37.23±0.72	43.62±0.36	51.04±0.1	51.8±0.18	35.83±0.58	45.48±0.55	45.38±0.54	46.78±0.68
Monounsaturated								
14:1*n*-7	0.09±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.21±0.02	0.1±0.01	0.1±0.01	0.11±0.01
15:1*n*-7	0.02±0	0.26±0.03	0.21±0.02	0.22±0.02	0.001±0	0.19±0.02	0.18±0.02	0.19±0.02
16:1*n*-7 *trans*	ND	ND	ND	ND	ND	ND	ND	0.01±0
16:1*n*-7	13.4±0.34	12.69±0.27	11.65±0.17	13.55±0.36	14.01±0.04	13.42±0.34	13.56±0.36	13.65±0.37
18:1*n*-9 *trans*	ND	ND	ND	ND	ND	ND	ND	ND
18:1*n*-9	13.08±0.31	12.89±0.29	11.01±0.01	10±0.01	12.01±0.02	12.48±0.25	12.23±0.22	12.14±0.21
20:1*n*-9	0.51±0.05	0.64±0.06	0.67±0.07	0.69±0.07	0.85±0.09	0.67±0.07	0.48±0.05	0.44±0.04
22:1*n*-9	2.49±0.25	2.21±0.22	2.23±0.22	2.23±0.22	2.65±0.27	2.06±0.21	1.94±0.19	1.31±0.13
24:1*n*-9	1.39±0.14	0.99±0.1	0.98±0.1	0.98±0.1	0.42±0.04	0.44±0.04	0.61±0.06	0.7±0.07
∑MUFA	30.98±0.01	29.78±0.98	26.85±0.69	27.77±0.78	30.151±0.02	29.36±0.94	29.1±0.91	28.55±0.86
Polyunsaturated								
18:2*n*6 *trans*	0.09±0.01	0.02±0	0.001±0	0.02±0	0.17±0.02	0.03±0	0.01±0	ND
18:2*n*-6	2.36±0.24	2.02±0.2	2.12±0.21	4.84±0.48	2.45±0.25	2.1±0.21	2.01±0.2	0.23±0.02
18:3*n*-6	0.67±0.07	0.65±0.07	0.56±0.06	0.55±0.06	0.49±0.05	0.43±0.04	0.44±0.04	0.26±0.03
18:3*n*-3	0.53±0.05	0.22±0.02	0.18±0.02	0.11±0.01	0.14±0.01	0.11±0.01	0.15±0.02	0.18±0.02
20:2*n*-6	3.19±0.32	2.87±0.29	1.74±0.17	1.05±0.11	2.87±0.29	2.1±0.21	2.06±0.21	2.04±0.2
20:3*n*-6	0.12±0.01	0.11±0.01	0.09±0.01	0.13±0.01	0.13±0.01	0.39±0.04	0.31±0.03	0.2±0.02
20:4*n*-6	1.34±0.13	0.99±0.1	1±0.1	0.87±0.09	0.84±0.08	0.19±0.02	0.27±0.03	0.37±0.04
20:5*n*-3 EPA	14.14±0.41	12.69±0.27	9.25±0.93	6.84±0.68*	15.21±0.52	12.52±0.25	12.7±0.27	12.91±0.29
22:5*n*-3	0.93±0.09	0.65±0.07	0.55±0.06	0.74±0.07	1.61±0.16	0.76±0.08	0.77±0.08	0.75±0.08
22:6*n*-3 DHA	4.61±0.46	4.01±0.4	4.55±0.46	3.47±0.43	4.81±0.42	4.77±0.48	4.38±0.44	4.2±0.42
∑PUFA	27.98±0.8	24.23±0.42	20.041±0.02	18.62±0.94*	28.72±0.81	23.4±0.34	23.1±0.31	21.14±0.11*
EPA+DHA	18.75±0.88	16.7±0.67	13.8±0.38	10.31±0.11*	20.2±0.94	17.29±0.73	17.08±0.71	17.11±0.71
∑*n*-3PUFA	20.21±0.02	17.57±0.76	14.53±0.45*	11.16±0.19*	21.77±0.12	18.16±0.82	18±0.08	18.04±0.8
∑*n*-6PUFA	7.68±0.77	6.64±0.66	5.51±0.55	7.44±0.74	6.78±0.68	5.21±0.52	5.09±0.51	3.1±0.31
∑*n*-3/∑*n*-6	2.63±0.26	2.65±0.27	2.64±0.26	1.5±0.16	3.21±0.31	3.49±0.35	3.54±0.35	5.82±0.58
∑*n*-6/∑*n*-3	0.38±0.04	0.38±0.04	0.38±0.04	0.67±0.06	0.31±0.03	0.29±0.03	0.28±0.03	0.17±0.02
∑PUFA/∑SFA	0.75±0.08	0.56±0.06	0.39±0.04	0.36±0.04	0.8±0.08	0.51±0.05	0.51±0.05	0.45±0.05
∑TRANS	0.09±0.01	0.02±0	ND	0.02±0	0.17±0.02	0.03±0	0.01±0	0.01±0

Data are exressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids Means with different superscripts (a, b, c, d) in the same row indicates a statistical difference ($p<0.05$). ND: not detected. ROG - RO + *H. valentiae* extract (0.5%), ROH - *J. rubens* extract (0.5%).

Table 7.3D Fatty acid composition of ROI and ROJ during accelerated storage for 12 days

	ROI				ROJ			
Days	**0**	**4**	**8**	**12**	**0**	**4**	**8**	**12**
Fatty acids								
Saturated								
14:0	13.61±0.36	12.33±0.23	13.38±0.34	16.25±0.63	13.17±0.32	10.11±0.01	11.26±0.13	12.38±0.24
15:0	0.86±0.09	0.89±0.09	0.96±0.1	1.14±0.11	0.82±0.08	0.83±0.08	0.91±0.09	0.89±0.09
16:0	20.37±0.03	21.96±0.2	24.05±0.41	25.44±0.54	19.85±0.99	19.04±0.9	21.26±0.13	23.68±0.37
17:0	1.08±0.11	1.06±0.11	1.12±0.11	1.29±0.13	0.97±0.1	0.98±0.1	1.01±0.1	1.07±0.11
18:0	4.64±0.45	4.58±0.46	5.05±0.51	6.05±0.61	7±0.7	4.96±0.5	4.77±0.48	4.78±0.48
20:0	0.33±0.03	0.36±0.04	1.01±0.1	1.19±0.12	0.19±0.02	0.29±0.03	0.48±0.05	0.97±0.1
22:0	0.1±0.01	0.03±0	0.2±0.02	0.53±0.05	0.21±0.02	0.2±0.02	0.21±0.02	0.14±0.01
24:0	0.03±0	0.03±0	0.05±0.01	0.09±0.01	0.04±0	0.06±0.01	0.07±0.01	0.08±0.01
∑SFA	41.02±0.1	41.24±0.12	45.82±0.58	51.98±0.2	42.25±0.23	36.47±0.65	39.97±0.04	43.99±0.04
Monounsaturated								
14:1*n*-7	0.22±0.02	0.1±0.01	0.1±0.01	0.11±0.01	0.08±0.01	0.08±0.01	0.08±0.01	0.09±0.01
15:1*n*-7	ND	0.03±0	0.02±0	0.03±0	0.02±0	0.03±0	0.03±0	0.04±0
16:1*n*-7 *trans*	ND	ND	0.01±0	0.01±0	ND	ND	ND	ND
16:1*n*-7	13.72±0.37	11.93±0.19	13.51±0.35	14.98±0.5	14.12±0.41	13.24±0.32	12.51±0.25	12.36±0.24
18:1*n*-9 *trans*	ND	ND	ND	ND	ND	ND	ND	ND
18:1*n*-9	11.56±0.16	10.32±0.03	10±0.01	9.86±0.99	13.36±0.34	12.56±0.26	10.41±0.04	9.7±0.97
20:1*n*-9	0.85±0.09	0.68±0.07	0.66±0.07	0.6±0.06	0.8±0.08	0.81±0.08	0.86±0.09	0.74±0.07
22:1*n*-9	2.77±0.28	2±0.2	2.01±0.2	1.19±0.12	2.68±0.27	2.44±0.24	2.31±0.23	2.23±0.22
24:1*n*-9	0.41±0.04	0.29±0.03	0.83±0.08	0.21±0.02	0.44±0.04	0.26±0.03	0.27±0.03	0.3±0.03
∑MUFA	29.53±0.95	25.35±0.54	27.14±0.71	26.99±0.7	31.5±0.15	29.42±0.94	26.47±0.65	25.46±0.05
Polyunsaturated								
18:2*n*6 *trans*	ND	ND	0.01±0	ND	0.18±0.02	0.02±0	0.01±0	0.01±0
18:2*n*-6	2.49±0.25	2.14±0.21	2.03±0.2	1.71±0.17	2.45±0.25	2.01±0.2	2.02±0.2	2.06±0.21
18:3*n*-6	0.48±0.05	0.41±0.04	0.42±0.04	0.47±0.05	0.97±0.1	0.98±0.1	0.99±0.1	0.45±0.05
18:3*n*-3	0.14±0.01	0.14±0.01	0.11±0.01	0.24±0.02	0.42±0.04	0.43±0.04	0.45±0.05	0.12±0.01
20:2*n*-6	2.86±0.29	2.18±0.22	2.07±0.21	1.09±0.11	2.84±0.28	2.21±0.22	2.01±0.2	2.19±0.22
20:3*n*-6	0.12±0.01	0.14±0.01	0.19±0.02	0.2±0.02	0.11±0.01	0.12±0.01	0.14±0.01	0.23±0.02
20:4*n*-6	0.79±0.08	0.09±0.01	0.63±0.06	0.47±0.05	0.12±0.01	0.11±0.01	0.11±0.01	0.65±0.07
20:5*n*-3 EPA	15.15±0.52	14.13±0.41	14.1±0.41	12.07±0.21	15.24±0.32	13.16±0.32	12.96±0.3	12.45±0.25
22:5*n*-3	1.51±0.15	1.31±0.13	0.71±0.07	0.57±0.06	1.12±0.11	1.26±0.13	0.96±0.1	0.85±0.09
22:6*n*-3 DHA	4.93±0.49	4.22±0.42	4.2±0.42	3.65±0.37	4.18±0.42	4.04±0.4	4.06±0.41	3.87±0.39
∑PUFA	28.47±0.85	24.76±0.48	24.47±0.45	20.47±0.05	27.63±0.56	24.34±0.43	23.71±0.37	22.88±0.29
EPA+DHA	20.08±0.01	18.35±0.84	18.3±0.83	15.72±0.57	19.42±0.74	17.2±0.72	17.02±0.7	16.32±0.63
∑*n*-3PUFA	21.73±0.17	19.8±0.98	19.12±0.91	16.53±0.65	20.96±0.09	18.89±0.89	18.43±0.84	17.29±0.73
∑n-6 PUFA	6.74±0.67	4.96±0.5	5.34±0.53	3.94±0.39	6.49±0.65	5.43±0.54	5.27±0.53	5.58±0.56
∑*n*-3/∑*n*-6	3.22±0.32	3.99±0.4	3.58±0.36	4.2±0.42	3.23±0.29	3.48±0.35	3.5±0.35	3.1±0.31
∑*n*-6/∑*n*-3	0.31±0.03	0.25±0.03	0.28±0.03	0.24±0.02	0.31±0.03	0.29±0.03	0.29±0.03	0.32±0.03
∑PUFA/∑SFA	0.69±0.07	0.6±0.06	0.53±0.05	0.39±0.04	0.65±0.06	0.67±0.07	0.59±0.06	0.52±0.05
∑TRANS	ND	ND	0.02±0	0.01±0	0.18±0.02	0.02±0	0.01±0	0.01±0

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fattyacids, ∑PUFA Total polyunsaturated fatty acids. Means with different superscripts (a, b, c, d) in the same row indicates a statistical difference ($p<0.05$). ND: not detected. ROI – RO + α-tocopherol (0.1%) and ROJ - RO + BHT (0.1%)

7.2.1D. Spectral Analysis

A comparison of ^{1}H-NMR spectrum of the refined oil (RO) and RO added with additives are shown in Fig. 7.5A&B. ROA at day 12 indicated the development of hydroperoxides (.OOH) and aldehydes (-CHO) due to the oxidation process during the accelerated stability study.

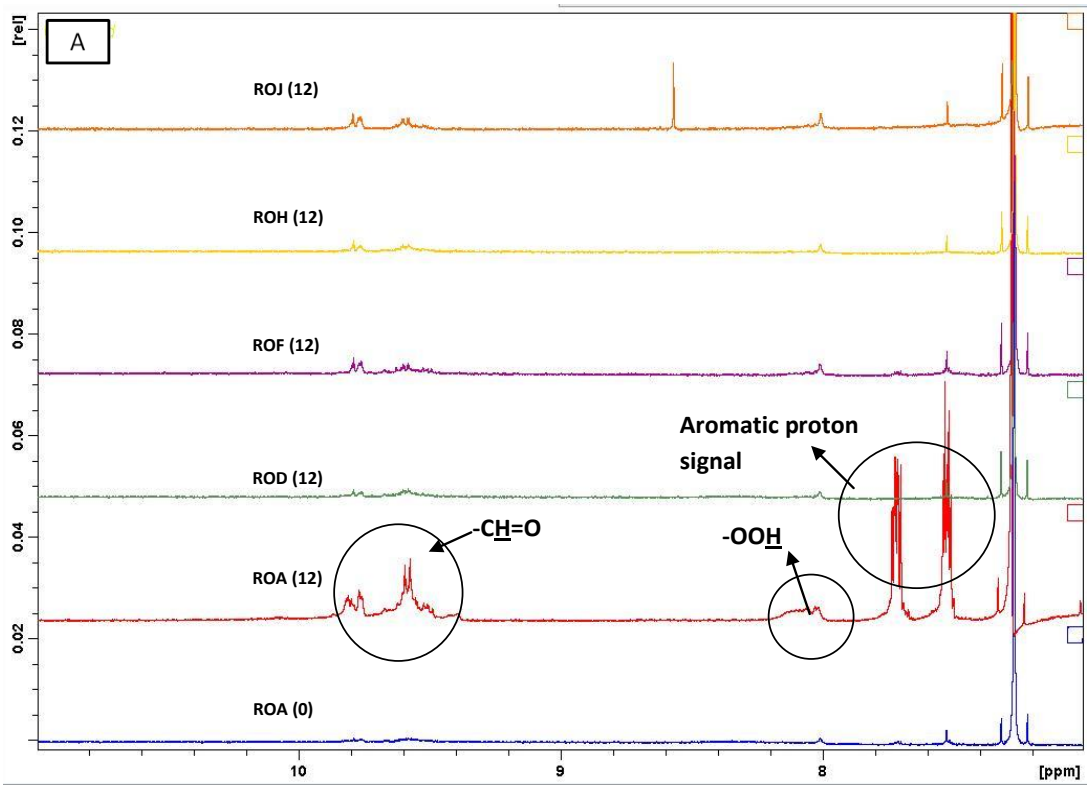


Fig. 7.5A. ^{1}H-NMR comparison of ROA, ROD, ROF, ROH, ROI and ROJ after 12 days of accelerated stability study of the refined oil, 11 – 7 ppm

It is further apparent from the ^{1}H-NMR spectrum as shown in Fig. 7.5A&B, that there was an increase in the aromatic proton integral after 12th day of accelerated stability study possibly due to the intra-molecular rearrangement/aromatisation reactions of the long chain unsaturated fatty acid system. This cyclisations are possibly due to the thermodynamical stability of this cyclic structure when heat/O_2 interferes in this system. However, ROD, ROF and ROH with potent antioxidant molecules showed no traces of aromatic protons δ 6.7 - 7.5 ppm, which indicate the property of these seaweed extracts to prevent the secondary intra-molecular

rearrangement/cyclisation/aromatisation reactions in the unsaturated system during storage.

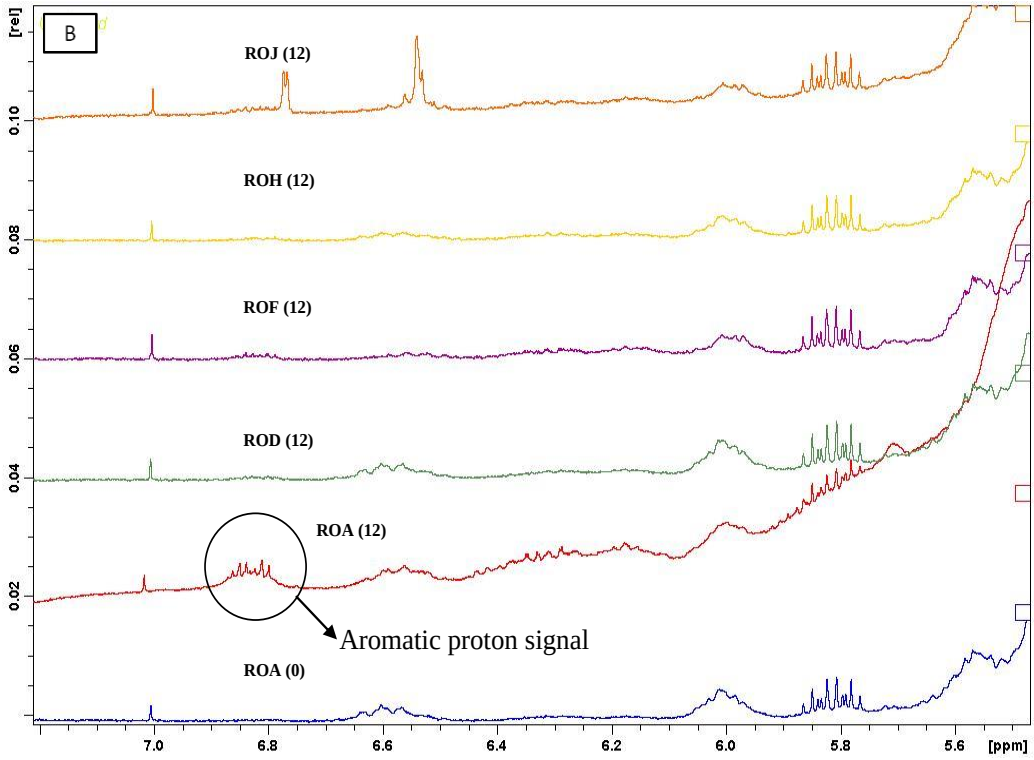
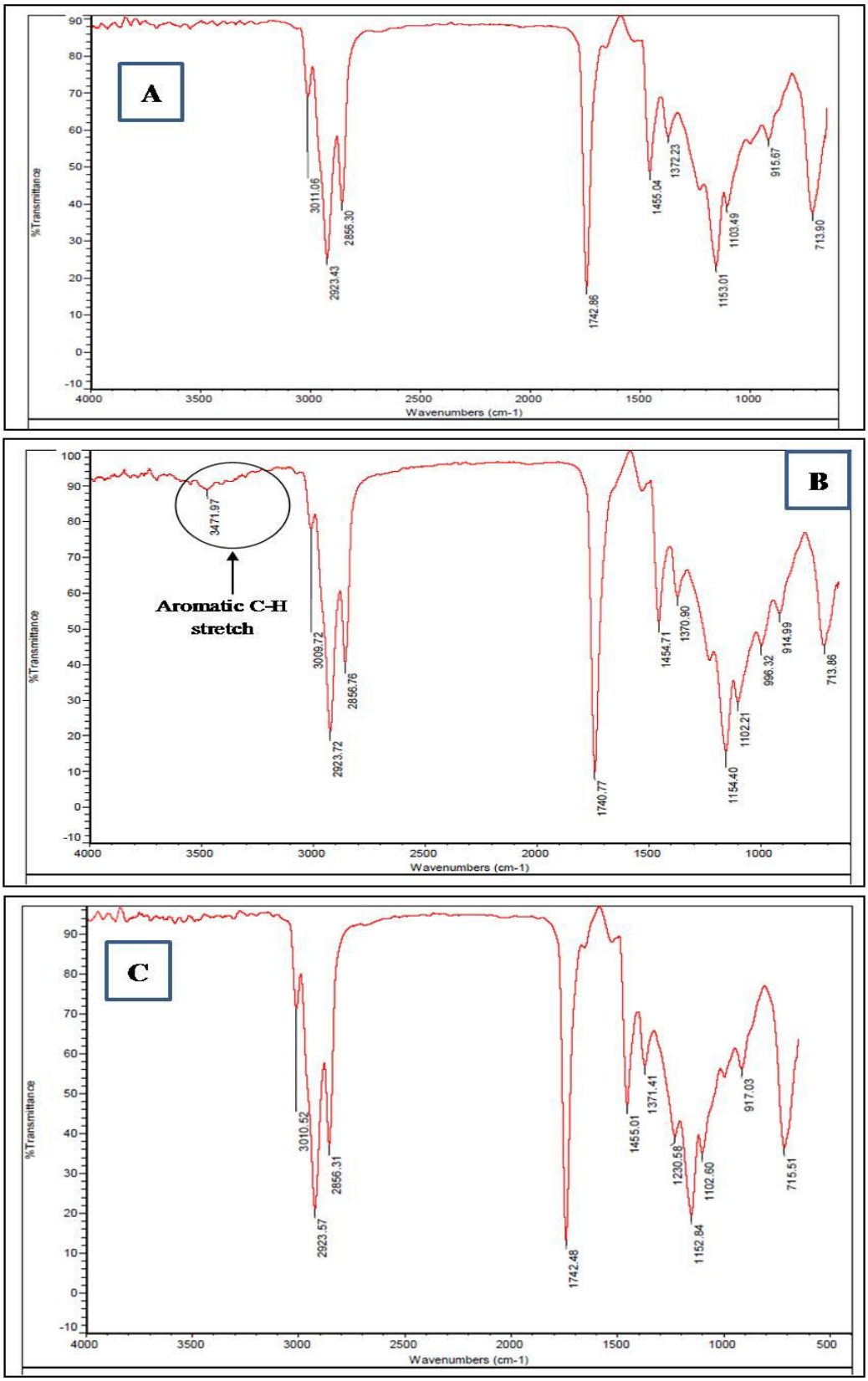


Fig. 7.5B. ^{1}H-NMR comparison of ROA, ROD, ROF, ROH, ROI and ROJ after 12 days of accelerated stability study of the refined oil, δ 8 – 5 ppm

The spectral analysis of the refined oil supplemented with various combinations of additives vis-à-vis without additives was further validated using FT-IR analysis. FT-IR spectra of the refined sardine oil with additives (ROD, ROF and ROH) after 12th day of the stability experiment revealed a significant reduction in C-H stretching vibrations (at 2900-3000 cm^{-1}), as compared to that in control, thereby explaining the absence of hydrocarbon impurities in the winterized oil. It is also interesting to note that the C-H stretching vibration was observed slightly higher than 3000 cm^{-1} (~ 3471 cm^{-1}) in the control after a period of 12 day of stability study, which led us to infer that at this region (Fig. 7.6A-E). Compounds that do not have aromatic bonds show C-H stretches only below 3000 cm^{-1}. The intensity of the carbonyl absorption at 1740 cm^{-1} was demonstrated to be intense in the ROA after 12th day of the stability experiment, whereas the signals of the additive supplemented treatment combinations exhibited medium or weak absorption.


A
%Transmittance
Wavenumbers (cm-1)
3011.06
2923.43
2856.30
1742.86
1455.04
1372.23
1153.01
1103.49
915.67
713.90
B
%Transmittance
Wavenumbers (cm-1)
3471.97
Aromatic C-H stretch
3009.72
2923.72
2856.76
1740.77
1454.71
1370.90
1154.40
1102.21
996.32
914.99
713.86
C
%Transmittance
Wavenumbers (cm-1)
3010.52
2923.57
2856.31
1742.48
1455.01
1371.41
1230.58
1152.84
1102.60
917.03
715.51

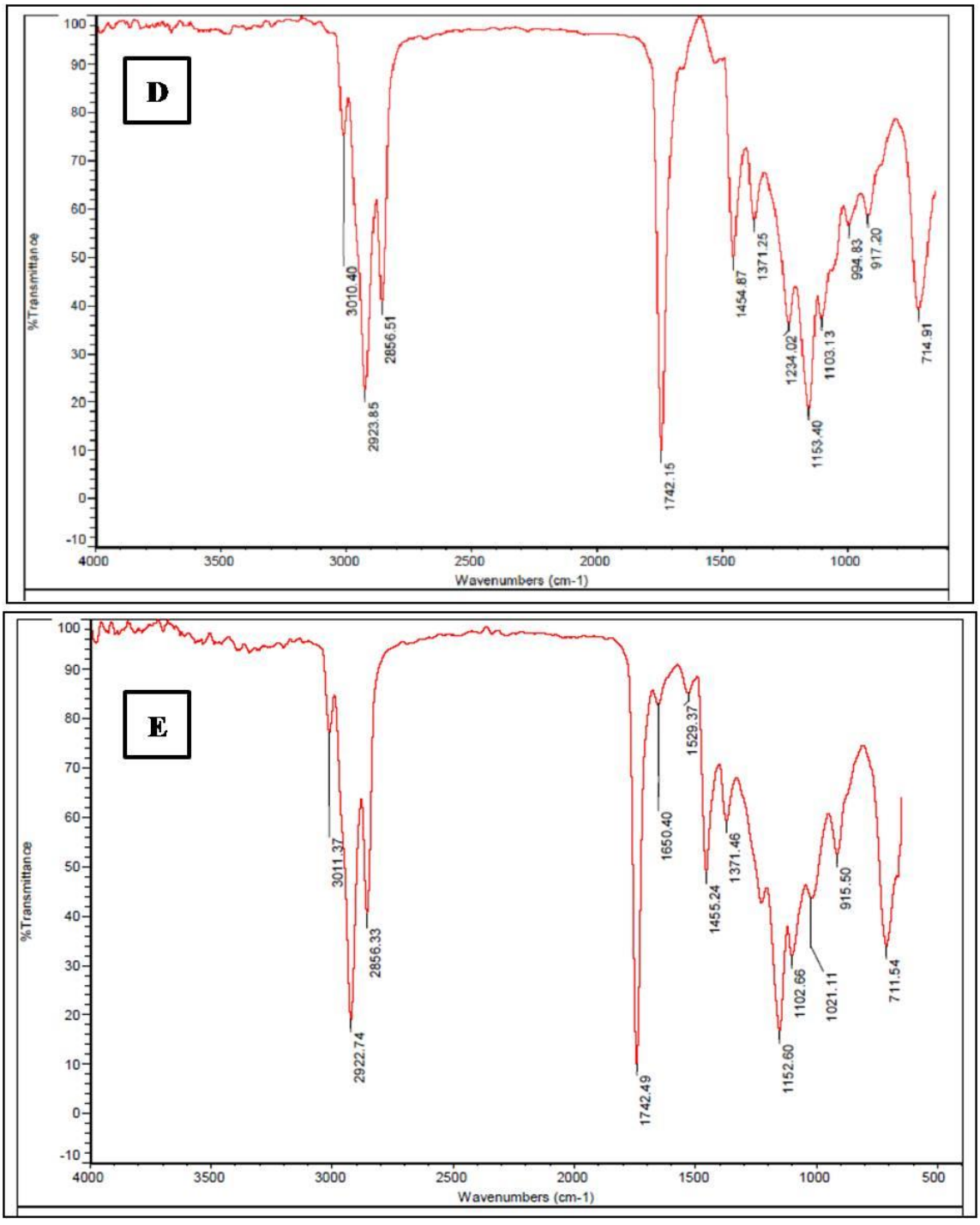


Fig.7.6. FTIR spectroscopic comparison of (**A**) refined oil (ROA) at 0th day, (**B**) refined oil (ROA) at 12th day, and refined oil added with various additives, (**C**) ROD at 12th day; (**D**) ROF at 12th day and (**E**) ROH at 12th day

7.2.2. Stability Study of Concentrated Sardine Fatty Acid Methyl Esters (CO)

7.2.2A. Oil stability Index using Rancimat Analysis

The effects of marine extract additives on the induction time of the concentrated sardine methyl esters (CO) are shown in Table 7.4 (Fig. 7.7.1 & 7.7.2). It was observed that the induction time was low for COA (0.13 h) as compared with that for refined oil (RO; 0.19 h). It can be due to the loss of natural antioxidants in the fish oil during the refining/concentration steps. The results showed that the extracts of marine origin (seaweeds and halophytic plants) exhibited significant antioxidant activities to prevent the degradation of unsaturated fatty acids in the concentrated fish methyl esters (CO). As it has been shown in Fig. 7.7.1, the lowest thermal stability in terms of induction time (IT) was for *S. maritima* extract (COB; 0.18 h). On the other hand, the highest IT was for the red seaweed *H. musciformis* extract (0.32 h), which was higher than the synthetic antioxidant BHT (0.27 h) and superior than the control COA (0.13 h). Thus, the antioxidative index (AAI) of the additives exhibited the order: COF > COD > COJ > COG ~ COH ~ COI > COC > COE > COB. This preliminary experiment using rancimat clearly revealed that the marine natural extracts, especially red seaweed extracts of *H. musciformis*, *K. alvarezii* and *J. rubens* possessed strong preventive capacity against the lipid peroxidation activity as compared to the control concentrated methyl esters. Interestingly, the extracts of *J. rubens* showed higher stability time (ST) of 1.74 h compared to that of BHT (0.62 h) and α-tocopherol (1.59 h). *H. musciformis* (ROF), *K. alvarezii* (ROD) also possessed higher IT than the synthetic antioxidant, α-tocopherol (0.25 h). In addition, *J. rubens* and *H. valentiae* showed similar IT to that of α-tocopherol. A similar study on sardine oil by Ganga *et al.,* (1998) showed no significant change in the induction period of sardine concentrate by the addition of α-tocopherol, while the synthetic antioxidants BHT and BHA significantly increased the induction period to 1.7–1.8 h ($p<0.05$).

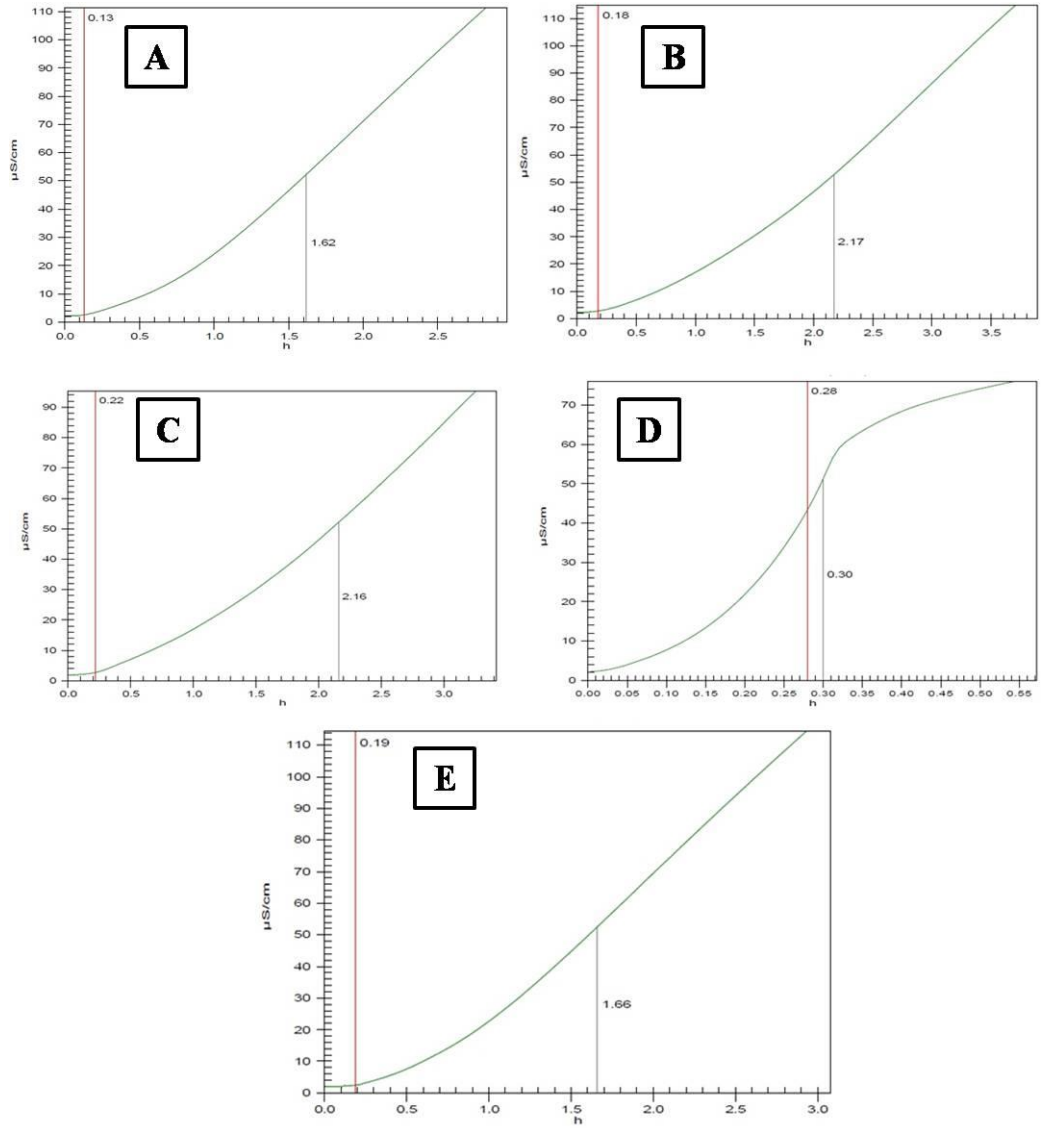
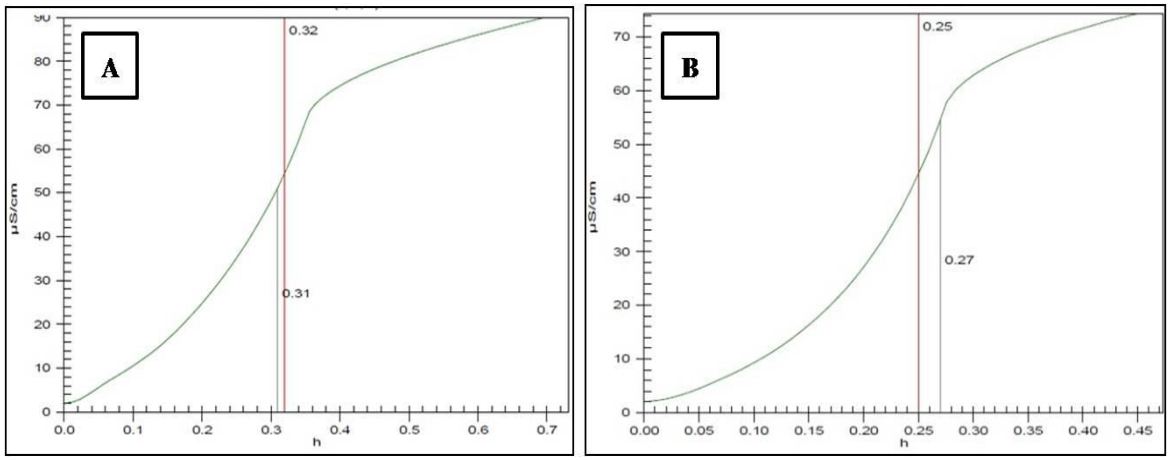


Fig. 7.7.1 Oil stability indices of (**A**) concentrated methyl esters COA and concentrated methyl esters added with various marine additives (**B**) COB; (**C**) COC; (**D**) COD; (**E**) COE



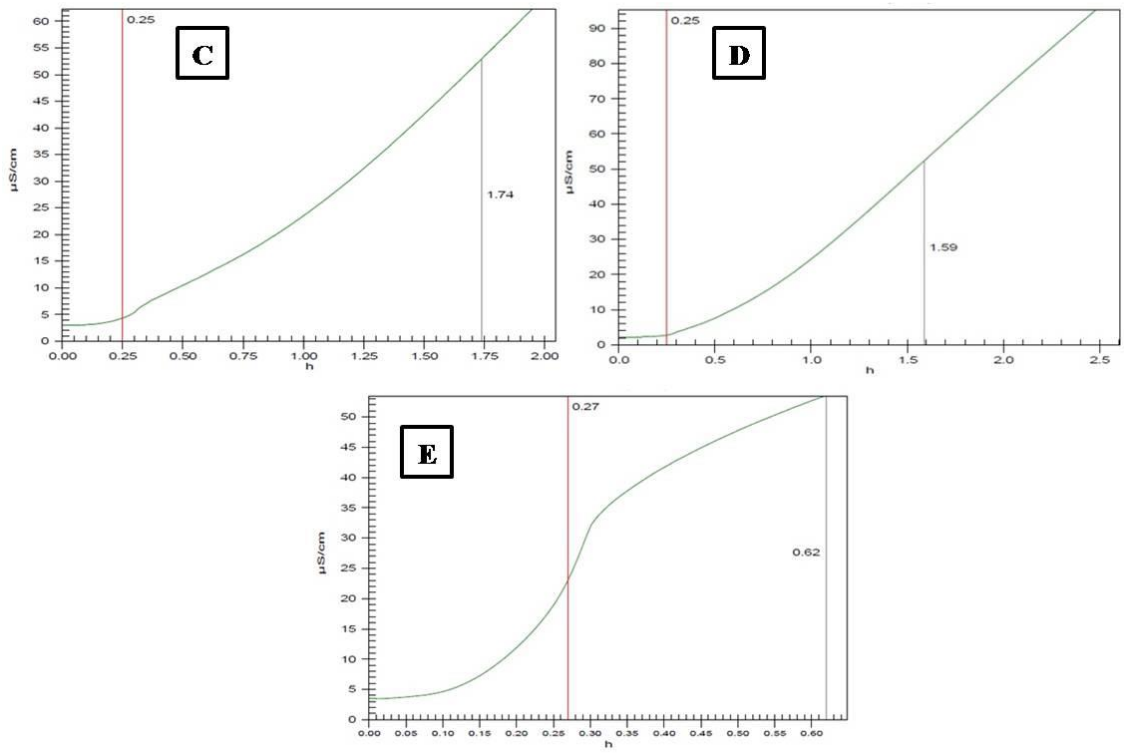


Fig. 7.7.2. Oil stability indices of (**A**) COF; (**B**) COG; (**C**) COH; (**D**) COI and (**E**) COJ

7.2.2B. P.V., pA.V., TOTOX, TBARS Values and DPPH Scavenging Activities during Accelerated Stability Study for 12 days

Primary oxidation products (hydroperoxides) were determined by P.V. measurement. In the absence of an antioxidant, the concentrated methyl esters deteriorated quickly, and the P.V. increased to 52.4 meqO_2/kg on the 4th day from 8.4 meqO_2/kg of 0th day (Table 7.4, Fig. 7.8). However, a rapid change in P.V. to 189.4 meqO_2/kg was observed for COA after 8th day. The P.V. of the COA increased 27-fold after 12 days to 228.1 meq O_2/kg. With the addition of 0.1 % BHT to the COA an increase of P.V. only upto 87.5 meqO_2/kg was observed (10.2 -fold). However, it is apparent that P.V. reached only upto 45.0 meqO_2/kg when *H. musciformis* was added to CO showing its best antioxidant index followed by COD and COH (51.3 & 60.2, respectively). The evaluation of P.V. showed that addition of marine derived antioxidants, particularly, *H. musciformis*, *J. rubens* and *K. alvarezzii* improved the shelf-life of CO and proved to be potent antioxidants than the synthetic antioxidants. This may be due to the action of phenolic compounds in these seaweeds. Phenolic

antioxidants (AOH) interfere with the oxidation of lipids and other molecules by rapid donation of hydrogen atom to the radicals (R) as follows (1):

$$AOH + R^{\cdot} \xrightarrow{1} AO^{\cdot} + RH$$

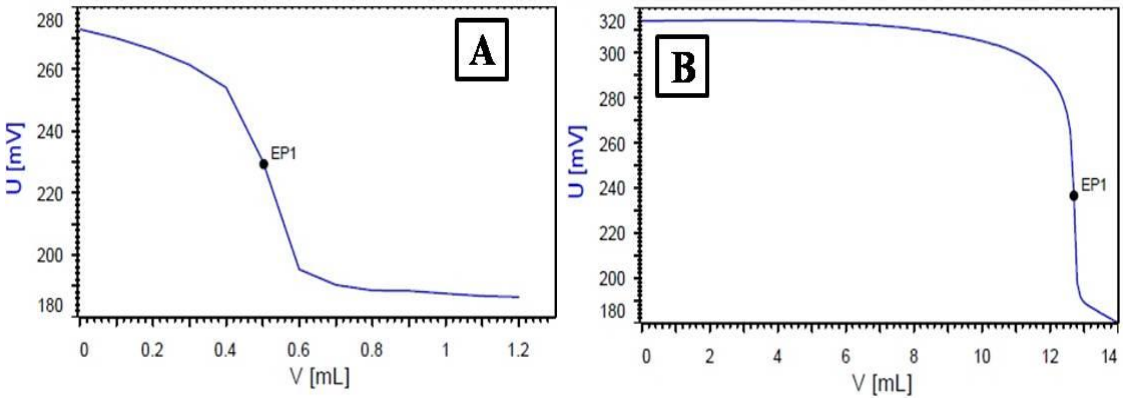
The phenoxy radical intermediates ($AO^{\cdot}$) are relatively stable due to resonance and therefore, a new chain reaction is not easily initiated. In addition, the phenoxy radical intermediates can also act as terminators of propagation route by reacting with other free radicals as follows (2):

$$AO^{\cdot} + R \xrightarrow{2} AOR$$

However, the additives of *S. brachiata*, *G. corticata* and *H. valentiae* exhibited *in vitro* antioxidant properties (*Chapter 6),* showed > 15 -fold increase in the P.V. value after 12 days of shelf-life study. Interestingly, *S. maritima* when added to CO after 12 days showed higher P.V. compared to CO (264.4 $meqO_2/kg$). This can be explained possibly due to their property to initiate an autoxidation process and act like prooxidants, under the heat stress conditions in the accelerated shelf life study. Thus, instead of terminating a free radical chain reaction by reacting with a second radical ($R^{\cdot}$) as shown above, the phenoxy radical ($AO^{\cdot}$) may also interact with O_2 to produce quinones (A=O) and superoxide anion ($O_2^{\cdot -}$) as shown below (Cotelle 2001):

$$AO^{\cdot} + O_2 \longrightarrow A{=}O + O_2^{\cdot -}$$

The small phenolic compounds which are easily oxidized, such as quercetin, gallic acid, possess prooxidant activity, as reported by (Hagerman *et al.*, 1998).



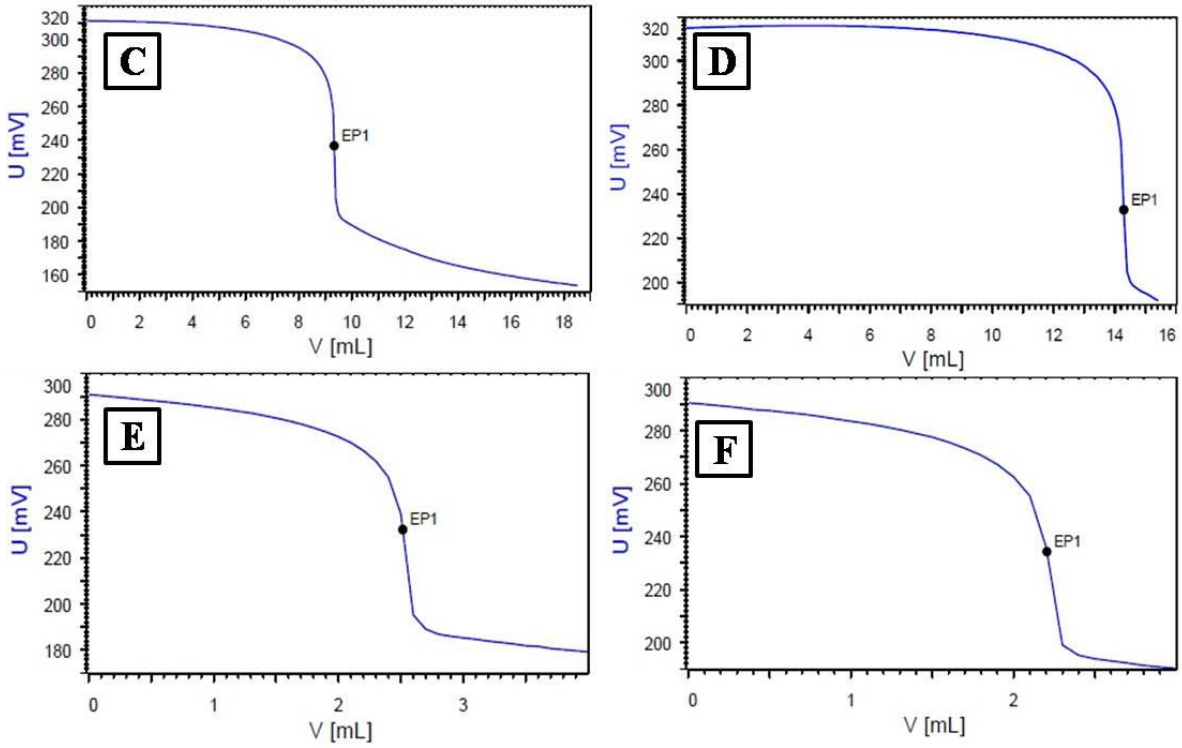


Fig. 7.8.1. Potentiometric titration curves obtained using an automatic titrator indicating the peroxide levels in concentrated methyl esters COA at (**A**) 0th day; (**B**) 12th day; (**C**) COB at 12th day; (**D**) COC at 12th day; (**E**) COD at 12th day; (**F**) RCOE at 12th day; (**G**) COF at 12th day and (**H**) COG at 12th day

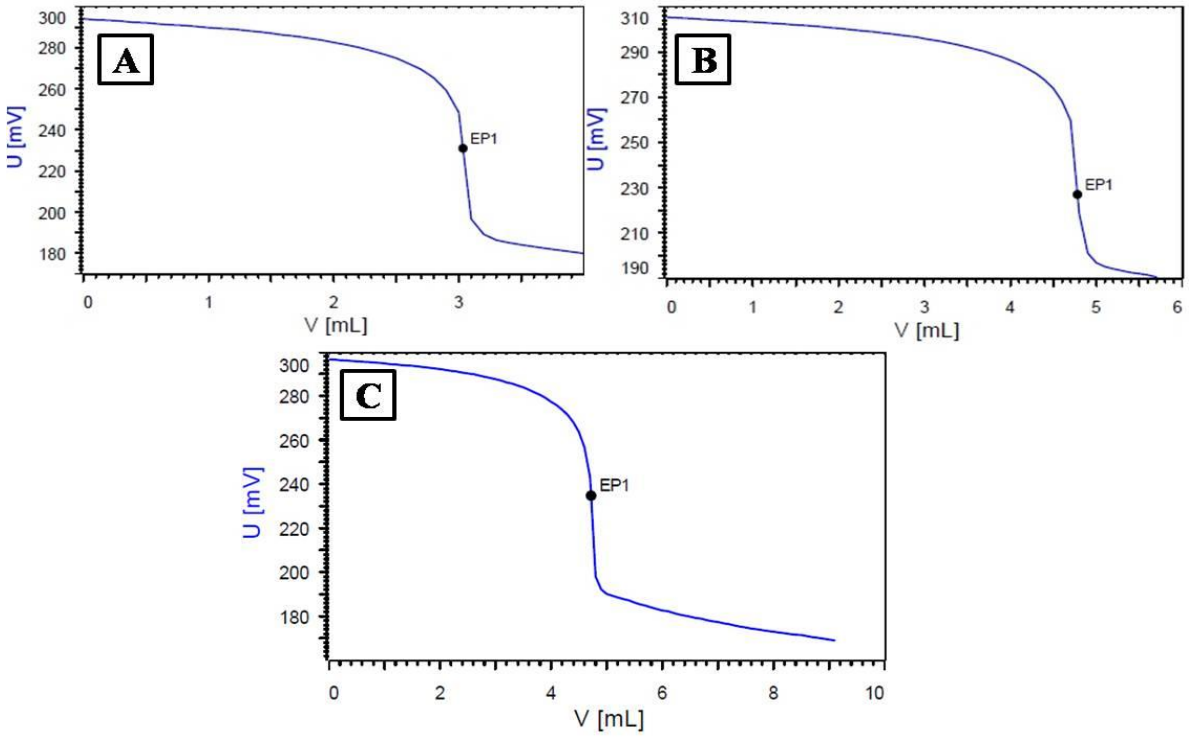


Fig. 7.8.2. Potentiometric titration curves obtained using an automatic titrator indicating the peroxide levels in; (**A**) COH; (**B**) COI and (**C**) COJ after 12 days of accelerated stability study

Formation of secondary oxidation products was expressed by an increase in p-anisidine values. Interestingly, similar to the concentrated sardine fatty acid methyl esters (CO), the halophyte extracts of *S. maritima* and *S. brachiata* showed lower pA.V. after 12 days of accelerated storage. This decrease in pA.V. may indicate that the secondary oxidation products have been further broken down after 12 days of storage. The pA.V. was found to increase more than 4-fold for COA, and lower than 2.4-fold increase for COD, COF and COH after 12 days (Table 7.4). These extracts may contain compounds that reduce the production of these secondary oxidation products. However, the synthetic antioxidants, α-tocopherol (COI) and BHT (COJ) showed 2.0 and 1.7 –fold, respectively in their pA.V. values after 12 days.

COA generally demonstrated significantly higher overall TOTOX values throughout the trial with more than 17.6 -fold increment after 12 days of storage (Table 7.4). The treatments COD, COF and COH exhibited increase in the TOTOX value iin the order, COH > COD > COF (5.6, 5.0, & 4.1 -fold, respectively), which demonstrate that the overall oxidation in the sardine methyl esters was reduced by the addition of the EtOAc fractions of *K. alvarezii, J. rubens* and *H. musciformis*. However, lower TOTOX value after the 12^{th} day on the addition of these marine extracts suggested that the seaweed antioxidants delay the oxidation process over time.

No significant differences in TBARS concentration among the treatments were discernable during the storage period, while COA showed significant change in its TBARS value with an increase upto 4.6 MDAEQ/kg from 1.6 MDAEQ/kg, which indicate the loss of the natural antioxidants in the concentrated fish fatty acid methyl esters. However, the TBARS values increased progressively over the entire storage period with less increase for COH followed by COF, COD ~ COJ, COI, COA, COE, COG and COC (Table 7.4). It is interesting that *J. rubens* and *H. musciformis* showed higher lipid peroxidation inhibitory ability than the synthetic antioxidant,

BHT. In addition, *K. alvarezii* showed comparable lipid peroxidation inhibitory ability to that of BHT (Table 7.4).

The DPPH scavenging activity of the COA reduced to 14.3 % from 79.4 % after 12 days of accelerated stability study. At 0^{th} day (d=0), the presence of free radical scavenging compounds in COA decreased the stability of DPPH radical showing higher activity of 79.4 %, while after 4^{th} day of storage, the alkoxyl ($RO^{\cdot}$), peroxyl ($ROO^{\cdot}$), or alkyl radicals ($R^{\cdot}$) from oxidized lipids which may reduce the absorbance of $DPPH^{\cdot}$ thereby showing higher activity (81 %) (Table 7.4). COD, COF and COH maintained > 60 % DPPH scavenging activity after 12 days of accelerated stability study comparable to that of synthetic antioxidants. This demonstrated that *K. alvarezii, H. musciformis* and *J. rubens* extracts possess antioxidative active principles.

Table 7.4 Characteristics of concentrated fatty acid methyl ester (COA), concentrated fatty acid methyl ester added with various marine extracts (COB - COH) and synthetic antioxidants (COI & COJ) during accelerated stability study at 65 °C

Parameters	Days	COA	COB	COC	COD	COE	COF	COG	COH	COI	COJ
IT		0.13	0.18	0.22	0.28	0.19	0.32	0.25	0.25	0.25	0.27
ST		1.62	2.17	2.16	0.3	1.66	0.31	0.27	1.74	1.59	0.62
AAI			1.38	1.69	2.15	1.46	2.46	1.92	1.92	1.92	2.08
P.V.	0	8.4±0.84^{a}	8.3±0.83^{a}	8.6±0.86^{a}	8.02±0.8^{a}	8.4±0.84^{a}	8.14±0.81^{a}	8.25±0.83^{a}	8.36±0.84^{a}	8.45±0.85^{a}	8.54±0.85^{a}
	4	52.36±0.24^{b}	46.21±0.62^{b}	44.36±0.44^{b}	27.67±0.77^{b}	46.24±0.62^{b}	22.26±0.23^{b}	34.46±0.45^{b}	26.31±0.63^{b}	28.26±0.83^{b}	20.43±0 .04^{b}
	8	189.36±0.94^{c}	134.31±0.43^{c}	186.37±0.64^{c}	44.97±0.5^{c}	116.35±0.64^{c}	32.44±0.24^{c}	67.21±0.72^{c}	37.26±0.73^{c}	53.47±0.35^{c}	38.43±0.84^{c}
	12	228.06±0.81^{d}	179.51±0.95^{d}	264.43±0.44^{d}	51.29±0.13^{d}	175.6±0.56^{d}	45.05±0.51^{d}	137.43±0.74^{d}	60.16±0.02^{d}	92.97±0.3^{d}	87.46±0.75^{d}
pA.V.	0	10±1^{a}	11.16±0.12^{a}	12.17±0.22^{a}	8.36±0.84^{a}	10.11±0.01^{a}	10.14±0.01^{a}	9.16±0.92^{a}	8.01±0.8^{a}	11.11±0.11^{a}	10.36±0.04^{a}
	4	20.06±0.01^{bd}	16.01±0.6^{a}	14.2±0.42^{a}	11.34±0.13^{a}	15.21±.52^{a}	13.31±0.33^{a}	14.49±0.45^{a}	12.43±0.24^{a}	17.01±0.7^{b}	14.21±0.42^{ab}
	8	24.07±0.41^{b}	18.11±0.81^{a}	25.43±0.54^{b}	14.21±0.42^{a}	20.36±0.04^{b}	15.64±0.56^{a}	22.21±0.22^{b}	14.45±0.45^{ab}	19.36±0.94^{bc}	15.41±0.54^{ab}
	12	15.47±0.55^{acd}	17.25±0.73^{a}	16.65±0.67^{a}	20.36±0.04^{b}	29.11±0.91^{c}	18.31±0.83^{a}	26.48±0.65^{b}	19.31±0.93^{b}	22.79±0.28^{c}	17.36±0.74^{b}
TOTOX	0	26.8±1.68^{a}	27.76±0.78^{a}	29.37±0.94^{a}	24.4±0.44^{a}	26.91±0.69^{a}	26.42±0.64^{a}	25.66±0.57^{a}	24.73±0.47^{a}	28.01±0.8^{a}	27.44±0.74^{a}
	4	124.78±0.48^{b}	108.43±0.84^{b}	102.92±0.29^{b}	66.68±0.67^{b}	107.69±0.77^{b}	57.83±0.78^{b}	83.41±0.34^{b}	65.05±0.51^{b}	73.53±0.35^{b}	55.07±0.51^{b}
	8	402.79±0.28^{c}	286.73±0.67^{c}	398.17±0.82^{c}	104.15±0.42^{c}	253.06±0.31^{c}	80.52±0.05^{c}	156.63±0.66^{c}	88.97±0.9^{c}	126.3±0.63^{c}	92.27±0.23^{c}
	12	471.59±0.16^{d}	376.27±0.63^{d}	545.51±0.55^{d}	122.94±0.29^{d}	380.31±0.03^{d}	108.41±0.84^{d}	301.34±0.13^{d}	139.63±0.96^{d}	208.73±0.87^{d}	192.28±0.23^{d}
TBARS	0	1.63±0.16^{a}	0.84±0.08^{a}	0.86±0.09^{a}	0.61±0.06^{a}	0.7±0.07^{a}	0.54±0.05^{a}	0.72±0.07^{a}	0.59±0.06^{a}	0.64±0.06^{a}	0.49±0.05^{a}
	4	2.84±0.28^{a}	0.91±0.09^{a}	0.94±0.09^{a}	0.74±0.07^{a}	0.76±0.08^{a}	0.61±0.06^{a}	0.96±0.1^{a}	0.64±0.06^{a}	0.76±0.08^{a}	0.69±0.07^{a}
	8	3.01±0.3^{a}	1.03±0.1^{a}	1.21±0.12^{a}	0.93±0.09^{a}	0.86±0.09^{a}	0.69±0.07^{a}	1.06±0.11^{a}	0.74±0.07^{a}	0.98±0.01^{a}	0.84±0.08^{a}
	12	4.63±0.46^{a}	1.26±0.13^{a}	2.87±0.29^{a}	1.01±0.01^{a}	1.94±0.19^{a}	0.99±0.01^{a}	2.01±0.2^{a}	0.97±0.01^{a}	1.09±0.11^{a}	1.01±0.01^{a}
DPPH	0	79.36±0.94^{a}	80.23±0.02^{a}	82.31±0.23^{a}	94.56±0.46^{a}	87.31±0.73^{a}	97.64±0.76^{a}	86.34±0.63^{a}	98.01±0.8^{a}	94.26±0.43^{a}	98.98±0.9^{a}
	4	80.99±0.01^{a}	71.64±0.16^{b}	60.39±0.04^{b}	78.64±0.86^{b}	56.23±0.62^{b}	76.48±0.65^{b}	61.49±0.15^{b}	79.31±0.93^{b}	80.26±0.03^{b}	90.38±0.04^{b}
	8	33.34±0.33^{b}	46.25±0.63^{c}	40.67±0.07^{c}	67.31±0.73^{b}	48.36±0.84^{b}	66.89±0.69^{c}	46.11±0.61^{c}	74.26±0.43^{bc}	65.34±0.53^{c}	83.99±0.4^{c}
	12	14.34±0.43^{c}	35.36±0.54^{d}	30.24±0.02^{d}	60.39±0.04^{c}	40.58±0.06^{c}	64.36±0.44^{c}	34.26±0.43^{d}	71.65±0.17^{c}	60.34±0.03^{c}	73.28±0.33^{d}

Data are expressed as mean ± standard deviation of three replicates. Means with different superscripts (a, b,c, d) in the column indicates a statistical difference ($p<0.05$).COA = CO Concentrated oil (Chapter 4) without any additives (Control); COB – CO + *S. brachiata* extract (0.5%), COC – CO + *S. maritima* extract (0.5%), COD – CO + *K. alvarezii* extract (0.5%), COE – CO + *G. corticata* extract (0.5%), COF – CO + *H. musciformis* extract (0.5%), COG - CO + *H. valentiae* extract (0.5%), COH – CO + *J. rubens* extract (0.5%), COI – CO + α-tocopherol (0.1%) and COJ - CO + BHT (0.1%); IT – Induction time in h; ST – Stability time in h; AAI - Antioxidant activity index = IT with antioxidant/IT without antioxidant; P.V. – Peroxide value represented in meqO_2/kg; pA.V. – p-anisidine value; TOTOX – total oxidation value (2 x P.V. + pA.V.); TBARS – Thiobarbituric acid reactive species represented in mg MDA equivalent compounds/kg sample (MDAEQ/kg);DPPH – free radical scavenging activity (%).

7.2.2C. Fatty acid Composition during Accelerated Shelf Life Study for 12 Days

A significant decrease in EPA and DHA was observed in the concentrated oil (% reduction 23.1 & 15.2 %, respectively) after storage period of 12 days due to the fact that LC-PUFA are more susceptible to oxidation than other fatty acids, such as SFAs or MUFAs (Table 7.5A-D). A decrease in the amount of these *n*-3 PUFAs over the storage period is an indication of oxidation of the methyl esters. *H. musciformis* and *J. rubens* extracts could significantly able to protect EPA (% reduction 16.7 & 21 %, respectively) and DHA (% reduction 3.5 & 6.5 %, respectively). BHT and α-tocopherol (COI & COJ) showed 14.1 & 17.2 % reduction, respectively in the EPA content after 12 days, while the DHA content reduced considerably for COI & COJ (13.4 & 20.8 % reduction, respectively). These results clearly indicated the potential of *H. musciformis* and *J. rubens* extracts to prevent oxidative degradation of the concentrated methyl esters. However, *G. corticata* extract (COE) was not as efficient radical scavenger as *H. musciformis* and *J. rubens* due to the significant reduction in EPA and DHA contents (21.2 & 14.8 % reduction, respectively) compared to COD (19.6 & 11 % reduction, respectively). It is of note that CO containing *S. brachiata* and *S. maritima* extracts (COB & COC) could able to prevent the oxidative degradation of EPA (32.3 & 37.2 % reduction, respectively) and DHA contents (20.6 & 19.1 % reduction, respectively) when compared to the control COA ($p<0.05$). It is interesting to note that *H. musciformis* extract prevent the reduction of *n*-3 PUFA compared to all other marine and synthetic additives.

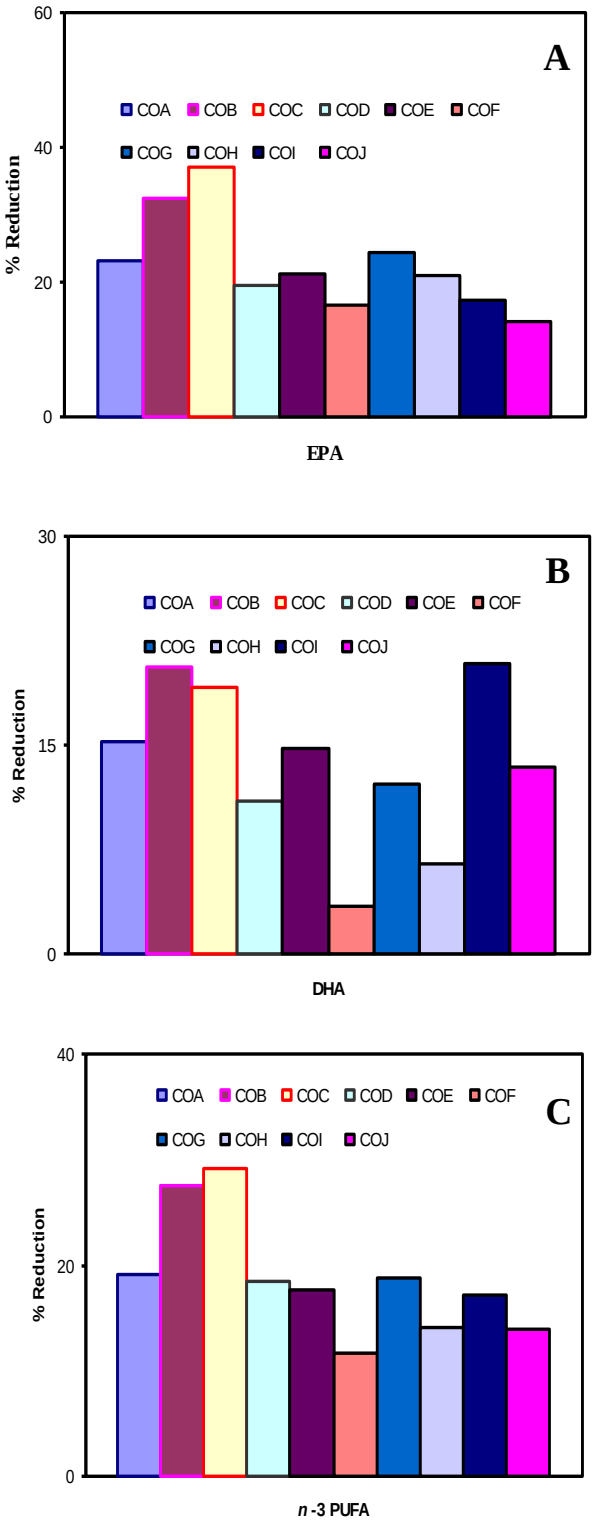


Fig. 7.9. Percent reduction in (**A**) EPA, (**B**) DHA and (**C**) ∑*n*-3PUFA after 12 days of accelerated shell life study of concentrated fatty acid methyl esters COA, CO added with various additives, COB-COH and synthetic additives (COI & COJ) compared to 0th day

Table 7.5 A Fatty acid composition of COA, COB and COC during accelerated storage for 12 days

	COA				COB				COC			
Days	0	4	8	12	0	4	8	12	0	4	8	12
Fatty acids												
Saturated												
14:0	0.97±0.1	1.01±0.1	1.23±0.12	2.94±0.29	0.98±0.1	0.98±0.1	0.97±0.1	0.99±0.1	0.96±0.1	0.97±0.1	0.98±0.1	1.01±0.1
15:0	0.16±0.02	0.16±0.02	0.15±0.02	0.13±0.01	0.17±0.02	0.16±0.02	0.15±0.02	0.18±0.02	0.16±0.02	0.15±0.02	0.16±0.02	0.18±0.02
16:0	0.64±0.06	0.56±0.06	0.13±0.01	0.05±0.01	0.65±0.07	0.67±0.07	0.63±0.06	0.63±0.06	0.66±0.07	0.75±0.08	0.64±0.06	0.31±0.03
17:0	0.47±0.05	0.51±0.05	0.48±0.05	0.51±0.05	0.51±0.05	0.51±0.05	0.52±0.05	1.02±0.1	0.52±0.05	0.57±0.06	0.58±0.06	0.52±0.05
18:0	1.94±0.19	2.03±0.2	2.36±0.24	0.13±0.01	1.94±0.19	2.01±0.2	2.65±0.27	3.69±0.37	1.86±0.19	1.99±0.2	2.01±0.2	2.61±0.26
20:0	0.1±0.01	0.01±0	0.02±0	0.02±0	0.1±0.01	0.13±0.01	0.16±0.02	0.12±0.01	0.14±0.01	0.16±0.02	0.26±0.03	0.46±0.05
22:0	0.08±0.01	0.03±0	0.03±0	0.03±0	0.09±0.01	0.1±0.01	0.14±0.01	0.85±0.09	0.09±0.01	0.16±0.02	0.09±0.01	0.08±0.01
24:0	0.13±0.01	0.13±0.01	0.16±0.02	0.11±0.01	0.14±0.01	0.11±0.01	0.12±0.01	0.89±0.09	0.13±0.01	0.14±0.01	0.05±0.01	0.16±0.02
∑SFA	4.49±0.45	4.44±0.44	4.56±0.46	3.92±0.39	4.58±0.46	4.67±0.47	5.34±0.53	8.37±0.84	4.52±0.45	4.89±0.49	4.77±0.48	5.33±0.53
Monounsaturated												
14:1*n*-7	0.1±0.01	0.14±0.01	0.15±0.02	0.15±0.02	0.11±0.01	0.12±0.01	0.12±0.01	0.13±0.01	0.12±0.01	0.12±0.01	0.09±0.01	0.09±0.01
15:1*n*-7	0.06±0.01	0.07±0.01	0.06±0.01	0.7±0.07	0.05±0.01	0.06±0.01	0.05±0.01	0.04±0	0.06±0.01	0.07±0.01	0.05±0.01	0.04±0
16:1*n*-7 *trans*	0.02±0	0.02±0	0.02±0	ND	0.01±0	0.01±0	0.02±0	0.02±0	0.02±0	0.01±0	0.1±0.01	0.01±0
16:1*n*-7	4.8±0.48	4.86±0.49	8.97±0.9	15.52±0.55	4.06±0.41	5.01±0.5	5.03±0.5	6.03±0.6	4.11±0.41	4.01±0.4	8.01±0.8	13.84±0.38*
18:1*n*-9 *trans*	0.01±0	0.01±0	0.01±0	ND	0.01±0	0.01±0	0.01±0	0.01±0	0.01±0	0.03±0	0.1±0.01	0.01±0
18:1*n*-9	5.74±0.57	5.76±0.58	6.01±0.6	6.19±0.62	5.81±0.58	5.76±0.58	5.78±0.58	5.98±0.6	5.76±0.58	5.85±0.59	5.91±0.59	5.96±0.6
20:1*n*-9	0.04±0	1.06±0.11	1.06±0.11	1.06±0.11	0.05±0.01	0.06±0.01	0.05±0.01	0.04±0	0.06±0.01	0.07±0.01	0.81±0.08	0.94±0.09
22:1*n*-9	4.34±0.43	4.13±0.41	4.23±0.42	4.43±0.44	4.06±0.41	4.26±0.43	4.35±0.44	4.22±0.42	4.09±0.41	4.19±0.42	4.36±0.44	5.04±0.5
24:1*n*-9	0.38±0.04	0.39±0.04	0.38±0.04	0.39±0.04	0.37±0.04	0.38±0.04	0.37±0.04	0.36±0.04	0.39±0.04	0.41±0.04	0.4±0.04	0.4±0.04
∑MUFA	15.49±0.55	16.44±0.64	20.89±0.09	28.44±0.84	14.53±0.45	15.67±0.57	15.78±0.58	16.83±0.68	14.62±0.46	14.76±0.48	19.83±0.98	26.33±0.63
Polyunsaturated												
18:2*n*6 *trans*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
18:2*n*-6	3.09±0.31	3.16±0.32	3.24±0.32	4.24±0.42	3.16±0.32	3.01±0.3	2.76±0.28	2.01±0.2	3.21±0.32	3.16±0.32	2.84±0.28	2.61±0.26
18:3*n*-6	0.59±0.06	0.51±0.05	0.52±0.05	0.33±0.03	0.61±0.06	0.52±0.05	0.58±0.06	0.31±0.03	0.61±0.06	0.71±0.07	0.7±0.07	0.76±0.08
18:3*n*-3	0.04±0	0.04±0	0.01±0	0.25±0.03	0.03±0	0.16±0.02	0.18±0.02	0.24±0.02	0.02±0	0.03±0	0.08±0.01	0.12±0.01
20:2*n*-6	5.9±0.59	4.96±0.5	4.86±0.49	4.77±0.48	5.94±0.59	5.01±0.5	4.53±0.45	4.26±0.43	4.74±0.47	4.73±0.47	4.51±0.45	4.03±0.4
20:3*n*-6	0.04±0	0.04±0	0.03±0	0.12±0.01	0.06±0.01	0.09±0.01	0.1±0.01	0.11±0.01	0.07±0.01	0.07±0.01	0.08±0.01	0.09±0.01
20:4*n*-6	1.12±0.11	1.1±0.11	1.1±0.11	1.1±0.11	1.04±0.1	1.05±0.11	1.06±0.11	1.08±0.11	1.01±0.1	1.01±0.1	1.01±0.1	1.04±0.1
20:5*n*-3 EPA	33.37±0.34	32.31±0.23	28.01±0.8	25.67±0.57*	34.01±0.4	28.31±0.83	26.34±0.63*	23.01±0.3*	33.46±0.35	33.03±0.3	26.2±0.62*	21.02±0.1*
22:5*n*-3	2.03±0.2	2.01±0.2	1.73±0.17	1.63±0.16	2.11±0.21	2.01±0.2	1.61±0.16	1.03±0.1	2.01±0.2	2±0.2	1.56±0.16	1.34±0.13
22:6*n*-3 DHA	27.76±0.78	26.41±0.64	25.32±0.53*	23.53±0.35	27.71±0.77	26.01±0.06	24.11±0.41	22.01±0.2*	27.05±0.71	26.86±0.69	23±0.3	21.87±0.19*
∑PUFA	73.94±0.39	70.54±0.05	64.82±0.48*	61.64±0.16*	74.67±0.47	66.17±0.62*	61.27±0.13*	54.06±0.41*	72.18±0.22	71.6±0.16	59.98±0.06*	52.88±0.29*
EPA+DHA	61.13±0.11	58.72±0.87	53.33±0.33*	49.2±0.92*	61.72±0.17	54.32±0.43*	50.45±0.05*	45.02±0.5*	60.51±0.05	59.89±0.99	49.2±0.92*	42.89±0.29*
∑*n*-3PUFA	63.2±0.32	60.77±0.08	55.07±0.51*	51.08±0.11*	63.86±0.39	56.49±0.65*	52.24±0.22*	46.29±0.63*	62.54±0.25	61.92±0.19	50.84±0.08*	44.35±0.44*
∑n-6PUFA	10.74±0.07	9.77±0.98	9.75±0.98	10.56±0.06	10.81±0.08	9.68±0.97	9.03±0.9	7.77±0.08	9.64±0.96	9.68±0.97	9.14±0.91	8.53±0.85
∑*n*-3/∑*n*-6	5.88±0.59	6.22±0.62	5.65±0.57	4.84±0.48	5.91±0.59	5.84±0.58	5.79±0.58	5.96±0.6	6.49±0.65	6.4±0.64	5.56±0.56	5.2±0.52
∑*n*-6/∑*n*-3	0.17±0.02	0.16±0.02	0.18±0.02	0.21±0.02	0.17±0.02	0.17±0.02	0.17±0.02	0.17±0.02	0.15±0.02	0.16±0.02	0.18±0.02	0.19±0.02
∑PUFA/∑SFA	16.47±0.65	15.89±0.59	14.21±0.42	15.72±0.57	16.3±0.63	14.17±0.42	11.47±0.15	6.46±0.65*	15.97±0.6	14.64±0.46	12.57±0.26	9.92±0.99*
∑TRANS	0.03±0	0.03±0	0.03±0	ND	0.02±0	0.02±0	0.03±0	0.03±0	0.03±0	0.04±0	0.2±0.02	0.02±0

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. Means with different superscripts (a, b, c, d) in the same row indicates a statistical difference ($p<0.05$). ND: not detected. COA = CO Concentrated oil (Chapter 4) without any additives (Control); COB – RO + *S. brachiata* extract (0.5%), COC – RO + *S. maritima* extract (0.5%).

Table 7.5B Fatty acid composition of COD, COE and COF during accelerated storage for 12 days

	COD				COE				COF			
Days	**0**	**4**	**8**	**12**	**0**	**4**	**8**	**12**	**0**	**4**	**8**	**12**
Fatty acids												
Saturated												
14:0	0.98±0.1	1.01±0.1	1.46±0.15	2.85±0.29	0.98±0.1	1.01±0.1	1.46±0.16	2.85±0.26	0.98±0.1	1.01±0.1	2.01±0.17	2.69±0.26
15:0	0.17±0.02	0.16±0.02	0.15±0.02	0.14±0.01	0.17±0.02	0.16±0.02	0.15±0.01	0.14±0.01	0.26±0.02	0.31±0.02	0.26±0.01	0.11±0.01
16:0	0.65±0.07	0.66±0.07	0.67±0.07	0.65±0.07	0.65±0.07	0.66±0.07	0.67±0.06	0.65±0.06	0.84±0.08	0.85±0.07	0.61±0.05	0.17±0.06
17:0	0.48±0.05	0.48±0.05	0.48±0.05	0.47±0.05	0.48±0.05	0.48±0.05	0.48±0.04	0.47±0.04	0.54±0.05	0.55±0.05	0.46±0.04	0.34±0.04
18:0	1.86±0.19	1.04±0.1	0.08±0.01	0.06±0.01	1.86±0.2	1.04±0.1	0.08±0.07	0.06±0.01	2.01±0.2	1.06±0.17	0.18±0.16	1.53±0.01
20:0	0.12±0.01	0.1±0.01	0.09±0.01	0.09±0.01	0.12±0.01	0.1±0.01	0.09±0.01	0.09±0.01	0.16±0.02	0.17±0.03	0.01±0.03	0.83±0.01
22:0	0.09±0.01	0.08±0.01	0.01±0	0.03±0	0.09±0.01	0.08±0.01	0.01±0	0.03±0	0.09±0.01	0.08±0.01	0.09±0.01	0.01±0.01
24:0	0.14±0.01	0.1±0.01	0.09±0.01	0.08±0.01	0.14±0.01	0.1±0.01	0.09±0.01	0.08±0.01	0.17±0.02	0.18±0.01	0.19±0.01	0.01±0.01
∑SFA	0.98±0.1	1.01±0.1	1.46±0.15	2.85±0.29	4.49±0.47	3.63±0.36	3.03±0.37	4.37±0.4	5.05±0.48	4.21±0.45	3.81±0.46	5.69±0.4
Monounsaturated												
14:1*n-7*	0.16±0.02	0.16±0.02	0.14±0.01	0.15±0.02	0.16±0.01	0.16±0.01	0.14±0.02	0.15±0.02	0.17±0.02	0.15±0.01	0.15±0.01	0.12±0.01
15:1*n-7*	0.11±0.01	0.16±0.02	0.14±0.01	0.18±0.02	0.11±0.01	0.16±0.01	0.14±0.01	0.18±0	0.09±0.01	0.06±0.01	0.05±0.05	0.52±0
16:1*n-7 trans*	0.01±0	ND	ND	ND	0.01±0	ND	ND	ND	ND	ND	ND	ND
16:1*n-7*	5.01±0.5	6.76±0.68	8.04±0.8	15.07±0.51	5.01±0.5	6.76±0.77	8.04±1	15.07±1.36	5.36±0.51	6.83±0.71	9.9±1.04	14.32±1.38
18:1*n-9 trans*	0.01±0	ND	0.01±0	ND	0.01±0	ND	0.01±0	ND	ND	ND	ND	ND
18:1*n-9*	5.86±0.59	5.71±0.57	5.56±0.56	5.62±0.56	5.86±0.6	5.71±0.63	5.56±0.69	5.62±0.77	6.26±0.6	6.01±0.57	5.98±0.59	5.98±0.59
20:1*n-9*	0.06±0.01	0.4±0.04	0.04±0	0.03±0	0.06±0.01	0.4±0.01	0.04±0.09	0.03±0.1	0.08±0.01	0.06±0.01	0.04±0	0.02±0
22:1*n-9*	4.51±0.45	4.55±0.46	4.56±0.46	4.58±0.46	4.51±0.45	4.55±0.4	4.56±0.41	4.58±0.44	4.91±0.48	4.9±0.49	4.9±0.49	4.93±0.48
24:1*n-9*	0.39±0.04	0.38±0.04	0.31±0.03	0.3±0.03	0.39±0.04	0.38±0.04	0.31±0.05	0.3±0.05	0.51±0.04	0.34±0.05	0.29±0.04	0.24±0.03
∑MUFA	16.12±0.61	18.12±0.81	18.8±0.88	25.93±0.59	16.12±1.61	18.12±1.87	18.8±2.25	25.93±2.74	17.38±1.67	18.35±1.85	21.31±2.23	26.13±2.51
Polyunsaturated												
18:2*n6 trans*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
18:2*n-6*	3.11±0.31	3.36±0.34	4.01±0.4	4.21±0.42	3.11±0.32	3.36±0.33	4.01±0.39	4.21±0.39	4.01±0.39	4.1±0.4	4.11±0.41	4.14±0.41
18:3*n-6*	0.62±0.06	0.96±0.1	1±0.1	1.07±0.11	0.62±0.06	0.96±0.09	1±0.2	1.07±0.03	0.76±0.07	0.78±0.08	1±0.08	0.98±0.11
18:3*n-3*	0.05±0.01	0.06±0.01	0.16±0.02	0.24±0.02	0.05±0.01	0.06±0.02	0.16±0.02	0.24±0.02	0.08±0.01	0.16±0.01	0.21±0.01	0.14±0.02
20:2*n-6*	6.01±0.6	5.41±0.54	4.96±0.5	4.87±0.49	6.01±0.6	5.41±0.53	4.96±0.49	4.87±0.47	5.88±0.61	5.46±0.6	5.24±0.58	5.1±0.51
20:3*n-6*	0.05±0.01	0.11±0.01	0.11±0.01	0.12±0.01	0.05±0	0.11±0.01	0.11±0.01	0.12±0.01	0.06±0.01	0.08±0.01	0.1±0.04	0.42±0.01
20:4*n-6*	1.01±0.1	1.06±0.11	1.04±0.1	1.12±0.11	1.01±0.1	1.06±0.1	1.04±0.12	1.12±0.11	1.01±0.1	1.05±0.11	1.1±0.11	1.05±0.12
20:5*n-3* EPA	34.64±0.46	30.31±0.03	28.65±0.87*	27.3±0.73*	34.64±3.42	30.31±2.86	28.65±2.72	27.3±2.66	33.65±3.53	31.14±3.33	28.31±3.02	28.04±2.67
22:5*n-3*	2.1±0.21	1.64±0.16	1.56±0.16	1.46±0.15	2.1±0.21	1.64±0.2	1.56±0.19	1.46±0.18	2.01±0.21	1.84±0.19	1.74±0.13	1.16±0.16
22:6*n-3* DHA	28.01±0.8	26.14±0.61	24.01±0.4	23.87±0.39*	28.01±2.81	26.14±2.6	24.01±2.5	23.87±2.46	27.65±2.9	26.31±2.8	26±2.71	26.69±2.55
∑PUFA	75.6±0.06	69.05±0.91*	65.5±0.55*	64.26±0.43*	75.6±7.53	69.05±6.73	65.5±6.65	64.26±6.33	75.11±7.83	70.92±7.53	67.81±7.08	67.72±6.55
EPA+DHA	62.65±0.27	56.45±0.65*	52.66±0.27*	51.17±0.12*	62.65±6.23	56.45±5.46	52.66±5.23	51.17±5.12	61.3±6.43	57.45±6.13	54.31±5.73	54.73±5.22
∑*n-3*PUFA	64.8±0.48	58.15±0.82*	54.38±0.44*	52.87±0.29*	64.8±6.45	58.15±5.68	54.38±5.43	52.87±5.32	63.39±6.65	59.45±6.33	56.26±5.86	56.03±5.4
∑n-6PUFA	10.8±0.08	10.9±0.09	11.12±0.11	11.39±0.14	10.8±1.08	10.9±1.05	11.12±1.22	11.39±1.01	11.72±1.18	11.47±1.2	11.55±1.21	11.69±1.15
∑*n-3*/∑*n-6*	6±0.6	5.33±0.53	4.89±0.49	4.64±0.46	6±0.6	5.33±0.54	4.89±0.45	4.64±0.53	5.41±0.57	5.18±0.53	4.87±0.48	4.79±0.47
∑*n-6*/∑*n-3*	0.17±0.02	0.19±0.02	0.2±0.02	0.22±0.02	0.17±0.02	0.19±0.02	0.2±0.02	0.22±0.02	0.18±0.02	0.19±0.02	0.21±0.02	0.21±0.02
∑PUFA/∑SFA	16.84±0.68	19.02±0.9	21.62±0.16*	14.7±0.47	16.84±1.6	19.02±1.87	21.62±1.81	14.7±1.59	14.87±1.64	16.85±1.68	17.8±1.53	11.9±1.62
∑TRANS	0.02±0	ND	0.01±0	ND	0.02±0	ND	0.01±0	ND	ND	ND	ND	ND

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. Means with different superscripts (a, b, c, d) in the same row indicates a statistical difference ($p<0.05$). ND: not detected. COD – CO + *K. alvarezii* extract (0.5%), COE – CO + *G. corticata* extract (0.5%), COF – CO + *H. musciformis* extract (0.5%).

Table 7.5C Fatty acid composition of COG and COH during accelerated storage for 12 days

	COG				COH			
Days	**0**	**4**	**8**	**12**	**0**	**4**	**8**	**12**
Fatty acids								
Saturated								
14:0	0.97±0.1	1±0.1	1.69±0.2	2.57±0.27	1.01±0.1	1.61±0.16	2.01±0.2	2.62±0.26
15:0	0.19±0.03	0.18±0.03	0.12±0.03	0.1±0.01	0.31±0.03	0.29±0.03	0.25±0.03	0.21±0.02
16:0	0.76±0.08	0.65±0.09	0.5±0.06	0.59±0.02	0.96±0.1	0.68±0.07	0.61±0.06	0.58±0.06
17:0	0.51±0.05	0.48±0.06	0.36±0.05	0.44±0.03	0.55±0.06	0.48±0.05	0.45±0.05	0.43±0.04
18:0	1.96±0.2	1.74±0.11	1.58±0.02	0.06±0.15	2.16±0.22	1.06±0.11	0.76±0.08	0.06±0.01
20:0	0.15±0.02	0.26±0.02	0.27±0	0.1±0.08	0.19±0.02	0.14±0.01	0.11±0.01	0.09±0.01
22:0	0.08±0.01	0.06±0.01	0.05±0.01	0.09±0	0.11±0.01	0.08±0.01	0.07±0.01	0.06±0.01
24:0	0.16±0.02	0.12±0.02	0.05±0.02	0.09±0	0.18±0.02	0.16±0.02	0.13±0.01	0.12±0.01
∑SFA	4.78±0.51	4.49±0.42	4.62±0.38	4.04±0.57	5.47±0.55	4.5±0.45	4.39±0.44	4.17±0.42
Monounsaturated								
14:1*n*-7	0.16±0.02	0.14±0.02	0.13±0.02	0.14±0.01	0.18±0.02	0.17±0.02	0.17±0.02	0.16±0.02
15:1*n*-7	0.08±0.01	0.09±0.01	0.46±0.01	0.03±0.05	0.09±0.01	0.11±0.01	0.15±0.02	0.16±0.02
16:1*n*-7 *trans*	0.01±0	ND	ND	ND	ND	ND	ND	ND
16:1*n*-7	5.13±0.54	7.14±0.68	10.44±0.99	13.83±1.43	5.41±0.54	6.86±0.69	8.94±0.89	13.85±1.39
18:1*n*-9 *trans*	ND	ND	ND	ND	ND	ND	ND	ND
18:1*n*-9	6.01±0.63	5.74±0.6	5.89±0.6	5.92±0.6	6.08±0.61	5.96±0.6	5.68±0.57	5.41±0.54
20:1*n*-9	0.07±0.01	0.06±0.01	0.03±0	0.04±0	0.07±0.01	0.05±0.01	0.04±0	0.03±0
22:1*n*-9	4.84±0.49	4.91±0.49	4.92±0.49	4.84±0.49	4.09±0.41	4.35±0.44	4.51±0.45	4.7±0.47
24:1*n*-9	0.41±0.05	0.45±0.03	0.4±0.03	0.29±0.02	0.54±0.05	0.51±0.05	0.49±0.05	0.47±0.05
∑MUFA	16.71±1.74	18.53±1.84	22.27±2.13	25.09±2.61	16.46±1.65	18.01±1.8	19.98±2	24.78±2.48
Polyunsaturated								
18:2*n*-6 *trans*	ND	ND	ND	ND	ND	ND	ND	ND
18:2*n*-6	3.91±0.4	4±0.41	4.1±0.41	4.11±0.41	4.06±0.41	4.07±0.41	4.07±0.41	4.07±0.41
18:3*n*-6	0.7±0.08	0.76±0.08	0.81±0.1	1.07±0.1	0.74±0.07	0.87±0.09	0.98±0.1	1.04±0.1
18:3*n*-3	0.06±0.01	0.1±0.02	0.11±0.02	0.23±0.01	0.09±0.01	0.16±0.02	0.21±0.02	0.23±0.02
20:2*n*-6	6.11±0.59	6.01±0.55	5.76±0.52	5.05±0.51	6.01±0.6	5.06±0.51	4.98±0.5	4.94±0.49
20:3*n*-6	0.05±0.01	0.14±0.01	0.41±0.01	0.12±0.04	0.08±0.01	0.09±0.01	0.1±0.01	0.1±0.01
20:4*n*-6	1.01±0.1	1.1±0.11	1.06±0.11	1.16±0.11	1.04±0.1	1.08±0.11	1.11±0.11	1.16±0.12
20:5*n*-3 EPA	35.31±3.4	33.3±3.11	30.16±2.83	26.69±2.8	34.21±3.59	33.14±3.31	31.24±3.12	27.03±2.7
22:5*n*-3	2.14±0.2	1.86±0.18	1.26±0.17	1.61±0.12	1.96±0.2	1.9±0.19	1.84±0.18	1.78±0.18
22:6*n*-3 DHA	29.01±2.89	28.01±2.63	27.11±2.6	25.46±2.67	27.42±2.79	26.16±2.62	25.98±2.6	25.65±2.57
∑PUFA	78.3±7.67	75.28±7.09	70.78±6.78	65.5±6.77	75.61±7.77	72.53±7.25	70.51±7.05	66±6.6
EPA+DHA	64.32±6.29	61.31±5.75	57.27±5.43	52.15±5.47	61.63±6.38	59.3±5.93	57.22±5.72	52.68±5.27
∑*n*-3PUFA	66.52±6.5	63.27±5.95	58.64±5.63	53.99±5.6	63.68±6.58	61.36±6.14	59.27±5.93	54.69±5.47
∑n-6PUFA	11.78±1.17	12.01±1.15	12.14±1.16	11.51±1.17	11.93±1.19	11.17±1.12	11.24±1.12	11.31±1.13
∑*n*-3/∑*n*-6	5.65±0.55	5.27±0.52	4.83±0.49	4.69±0.48	5.34±0.55	5.49±0.55	5.27±0.53	4.84±0.48
∑*n*-6/∑*n*-3	0.18±0.02	0.19±0.02	0.21±0.02	0.21±0.02	0.19±0.02	0.18±0.02	0.19±0.02	0.21±0.02
∑PUFA/∑SFA	16.38±1.52	16.77±1.69	15.32±1.78	16.21±1.19	13.82±1.42	16.12±1.61	16.06±1.61	15.83±1.58
∑TRANS	0.01±0	ND	ND	ND	ND	ND	ND	ND

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. Means with different superscripts (a, b, c, d) in the same row indicates a statistical difference ($p<0.05$). ND: not detected. COG - CO + *H. valentiae* extract (0.5%), COH – CO + *J. rubens* extract (0.5%).

Table 7.5D Fatty acid composition of COI and COJ during accelerated storage for 12 days

	COI				COJ			
Days	**0**	**4**	**8**	**12**	**0**	**4**	**8**	**12**
Fatty acids								
Saturated								
14:0	0.96±0.1	1.04±0.1	2.44±0.24	2.93±0.29	0.97±0.1	1.61±0.16	2.01±0.2	2.71±0.27
15:0	0.15±0.02	0.14±0.01	0.13±0.01	0.12±0.01	0.16±0.02	0.11±0.01	0.1±0.01	0.09±0.01
16:0	0.65±0.07	0.51±0.05	0.11±0.01	0.04±0	0.66±0.07	0.68±0.07	0.65±0.07	0.63±0.06
17:0	0.47±0.05	0.48±0.05	0.5±0.05	0.51±0.05	0.48±0.05	0.47±0.05	0.47±0.05	0.47±0.05
18:0	1.85±0.19	0.96±0.1	0.11±0.01	0.06±0.01	1.06±0.11	1±0.1	0.09±0.01	0.05±0.01
20:0	0.1±0.01	0.11±0.01	0.12±0.01	0.12±0.01	0.14±0.01	0.09±0.01	0.09±0.01	0.09±0.01
22:0	0.09±0.01	0.09±0.01	0.08±0.01	0.08±0.01	0.08±0.01	0.06±0.01	0.04±0	0.02±0
24:0	0.13±0.01	0.11±0.01	0.08±0.01	0.06±0.01	0.12±0.01	0.11±0.01	0.1±0.01	0.1±0.01
∑SFA	4.4±0.44	3.44±0.34	3.57±0.36	3.92±0.39	3.67±0.37	4.13±0.41	3.55±0.36	4.16±0.42
Monounsaturated								
14:1 *n-7*	0.1±0.01	0.12±0.01	0.16±0.02	0.19±0.02	0.11±0.01	0.11±0.01	0.12±0.01	0.13±0.01
15:1 *n-7*	0.06±0.01	0.05±0.01	0.05±0.01	0.04±0	0.07±0.01	0.06±0.01	0.05±0.01	0.04±0
16:1 *n-7 trans*	0.01±0	0.01±0	0.01±0	ND	ND	ND	ND	ND
16:1 *n-7*	4.7±0.47	5.86±0.59	10.26±0.03	15.11±0.51	4.97±0.5	6.24±0.62	10.46±0.05	14.27±0.43
18:1 *n-9 trans*	0.01±0	ND	ND	ND	ND	ND	ND	ND
18:1 *n-9*	5.75±0.58	6.46±0.65	7.81±0.78	8.06±0.81	5.87±0.59	6.16±0.62	7.11±0.71	7.97±0.8
20:1 *n-9*	0.05±0.01	0.69±0.07	0.91±0.09	1.05±0.11	0.04±0	0.03±0	0.03±0	0.04±0
22:1 *n-9*	4.36±0.44	4.38±0.44	4.4±0.44	4.41±0.44	4.41±0.44	4.51±0.45	4.52±0.45	4.55±0.46
24:1 *n-9*	0.39±0.04	0.26±0.03	0.2±0.02	0.17±0.02	0.36±0.04	0.26±0.03	0.22±0.02	0.13±0.01
∑MUFA	15.43±0.54	17.83±0.78	23.8±0.38	29.03±0.09	15.83±0.58	17.37±0.74	22.51±0.25	27.13±0.07
Polyunsaturated								
18:2 *n-6 trans*	ND	ND	ND	ND	ND	ND	ND	ND
18:2 *n-6*	3.11±0.31	3.16±0.32	3.28±0.33	4.09±0.41	3.86±0.39	4±0.4	4.06±0.41	4.08±0.41
18:3 *n-6*	0.61±0.06	0.5±0.05	0.46±0.05	0.3±0.03	0.71±0.07	0.86±0.09	1.01±0.1	1.03±0.1
18:3 *n-3*	0.04±0	0.16±0.02	0.18±0.02	0.23±0.02	0.05±0.01	0.16±0.02	0.18±0.02	0.23±0.02
20:2 *n-6*	5.81±0.58	5.14±0.51	4.97±0.5	4.7±0.47	5.91±0.59	5.26±0.53	4.94±0.49	4.8±0.48
20:3 *n-6*	0.05±0.01	0.07±0.01	0.09±0.01	0.1±0.01	0.06±0.01	0.09±0.01	0.1±0.01	0.11±0.01
20:4 *n-6*	1.09±0.11	1.08±0.11	1.07±0.11	1.06±0.11	1.21±0.12	1.16±0.12	1.14±0.11	1.11±0.11
20:5 *n-3* EPA	33.36±0.34	30.24±0.02	29.26±0.93	27.61±0.76*	32.01±0.2	30.11±0.01	28.41±0.84	27.49±0.75*
22:5 *n-3*	2.04±0.2	2.14±0.21	2.36±0.24	2.51±0.25	2.06±0.21	2±0.2	1.86±0.19	1.52±0.15
22:6 *n-3* DHA	27.94±0.79	25.14±0.51	23.11±0.31*	22.12±0.21*	26.26±0.63	24.11±0.41	23.21±0.32	22.74±0.27*
∑PUFA	74.05±0.41	67.63±0.76*	64.78±0.48*	62.72±0.27*	72.13±0.21	67.75±0.78*	64.91±0.49*	63.11±0.31*
EPA+DHA	61.3±0.13	55.38±0.54*	52.37±0.24*	49.73±0.97*	58.27±0.83	54.22±0.42	51.62±0.16*	50.23±0.02*
∑*n-3*PUFA	63.38±0.34	57.68±0.77*	54.91±0.49*	52.47±0.25*	60.38±0.04	56.38±0.64	53.66±0.37*	51.98±0.2*
∑n-6PUFA	10.67±0.07	9.95±0.01	9.87±0.99	10.25±0.03	11.75±0.18	11.37±0.14	11.25±0.13	11.13±0.11
∑*n-3*/∑*n-6*	5.94±0.59	5.8±0.58	5.56±0.56	5.12±0.51	5.14±0.51	4.96±0.5	4.77±0.48	4.67±0.47
∑*n-6*/∑*n-3*	0.17±0.02	0.17±0.02	0.18±0.02	0.2±0.02	0.19±0.02	0.2±0.02	0.21±0.02	0.21±0.02
∑PUFA/∑SFA	16.83±0.68	19.66±0.97	18.15±0.82	16±0.06	19.65±0.97	16.4±0.64	18.28±0.83	15.17±0.52
∑TRANS	0.02±0	0.01±0	0.01±0	ND	ND	ND	ND	ND

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. Means with different superscripts (a, b, c, d) in the same row indicates a statistical difference ($p<0.05$). ND: not detected. COI – CO + α-tocopherol (0.1%) and COJ - CO + BHT (0.1%)

7.2.3. Effect of different treatments (COT_{1-9}) on the stability of concentrated methyl esters (CO)

7.2.3A. Oil Stability Index using Rancimat Analysis

The effects of different treatments of marine extract combinations on the induction times for CO are shown in Table 7.6 (Fig. 7.10). The induction time (IT) of the various treatments were in the order, COT_3 (1.61) > COT_8 (1.06) > COT_5 (0.98) > COT_1 (0.61) > COT_2 (0.58) > COT_7 (0.56) > COT_9 (0.54) ~ COT_6 (0.54) > COT_4 (0.45). The combinations, COT_3 and COT_8 showed 6.0 and 4.6-fold increment in IT (Table 7.6) than the synthetic antioxidant, BHT (COJ, Table 7.4). The negative values observed for % synergism (%SYN) for COT_2, COT_4, COT_6, COT_7 and COT_9 clearly indicates the antagonistic effect of these treatments. It is apparent that in all these treatments the amount of *H. musciformis* extract was low (≤ 0.1%) as compared to other treatments. However, the higher percent synergism was observed for COT_3 followed by COT_8, COT_5 and COT_1 (Table 7.6). The maximum positive synergy was observed for COT_3 (221.7 %SYN) containing *K. alvarezii*, *H. musciformis* and *J. rubens* in the ratios 0.1:0.2:0.2. The extracts of *K. alvarezii*, *H. musciformis* and *J. rubens* added in the ratios of 0.4:0.5:0.5 (COT_7), 0.05:0.05:0.4 (COT_9), 0.2:0.1:0.2 (COT_2), 0.3:0.1:0.1 (COT_4) and 0.1:0.1:0.3 (COT_6) resulted in antagonism (negative % SYN), which contradict with the induction times. The antioxidant synergy based on the studies of Erhan *et al*. (2006) and Miranova *et al*. (2008) can be proposed by the hydrogen donation of the more active antioxidant to regenerate the other antioxidant and the formation of heterodimer from the moieties of the antioxidant during process of autoxidation. The individual antioxidant effects of *K. alvarezii*, *H. musciformis* and *J. rubens* extracts and the synergism exhibited by these two natural substances to prevent the oxidation of PUFA ester concentrate, are good examples of the effectiveness of these seaweed extracts. These seaweeds may act by scavenging free radicals or excited forms of oxygen that are involved in the initial steps of fatty acid oxidation. Ganga et al (1998) observed that quercetin and boldine, added singly to the sardine methyl ester concentrate, showed good inhibitory effects towards oxidation, but when these two natural products were assayed as a mixture, induction periods were

significantly increased. The earlier study of Yi *et al.,* (1991) also showed that the antioxidant effect of a ternary combination of compounds (ascorbic acid, lecithin and δ-tocopherol) in fish methyl esters was evidenced at high temperatures (80 °C). Miranova *et al.,* (2008) reported that mixtures of α-tocopherol and myricetin produced a synergistic effect during the autoxidation of triglycerols of sunflower oil, where the best interaction was achieved using equal molar ratios of the antioxidants at concentrations lower than 0.001 M. Becker *et al.,* (2007) showed that binary combinations of four antioxidants (α-tocopherol, astaxanthin, quercetin and rutin) may affect the synergism of antioxidant blends. Similarly, Niki *et al.,* (1984) demonstrated synergism between α-tocopherol and ascorbic acid in methyl linoleate, due to the regeneration of α-tocopherol by ascorbic acid. In addition, mutual synergistic effects were also observed between different phenolic compounds or with other non-phenolic antioxidants (Vinson *et al.,* 2001) and, in general, a combination of phenolic or other antioxidants exert better antioxidant effect than pure individual compound.

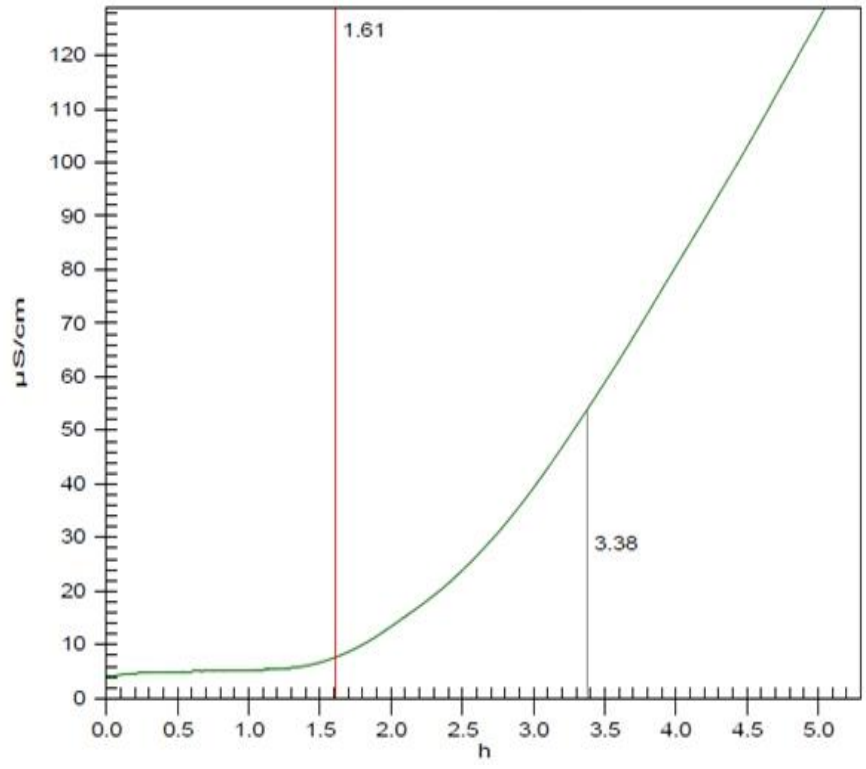


Fig.7.10. An indicative rancimat curve showing the oil stability indices of the treatment, COT_3

7.2.3B. P.V., pA.V., TOTOX Values, TBARS Values & DPPH Scavenging Activities during Accelerated Stability Study of 12 days

P.V. was found to be lowest for COT_3, followed by COT_8, COT_5, COT_1, COT_2, COT_4, COT_6, COT_9 and COT_7 after 12 days of shelf-life study (Table 7.6) (Fig. 7.11). The treatments, COT_4, COT_6, COT_7, and COT_9 exhibited more than 7.6 – fold reduction in their P.V. values whereas, the treatment combinations COT_1, COT_2, COT_3, COT_5 and COT_8 showed 4 to 5 -fold reduction in their peroxide values.

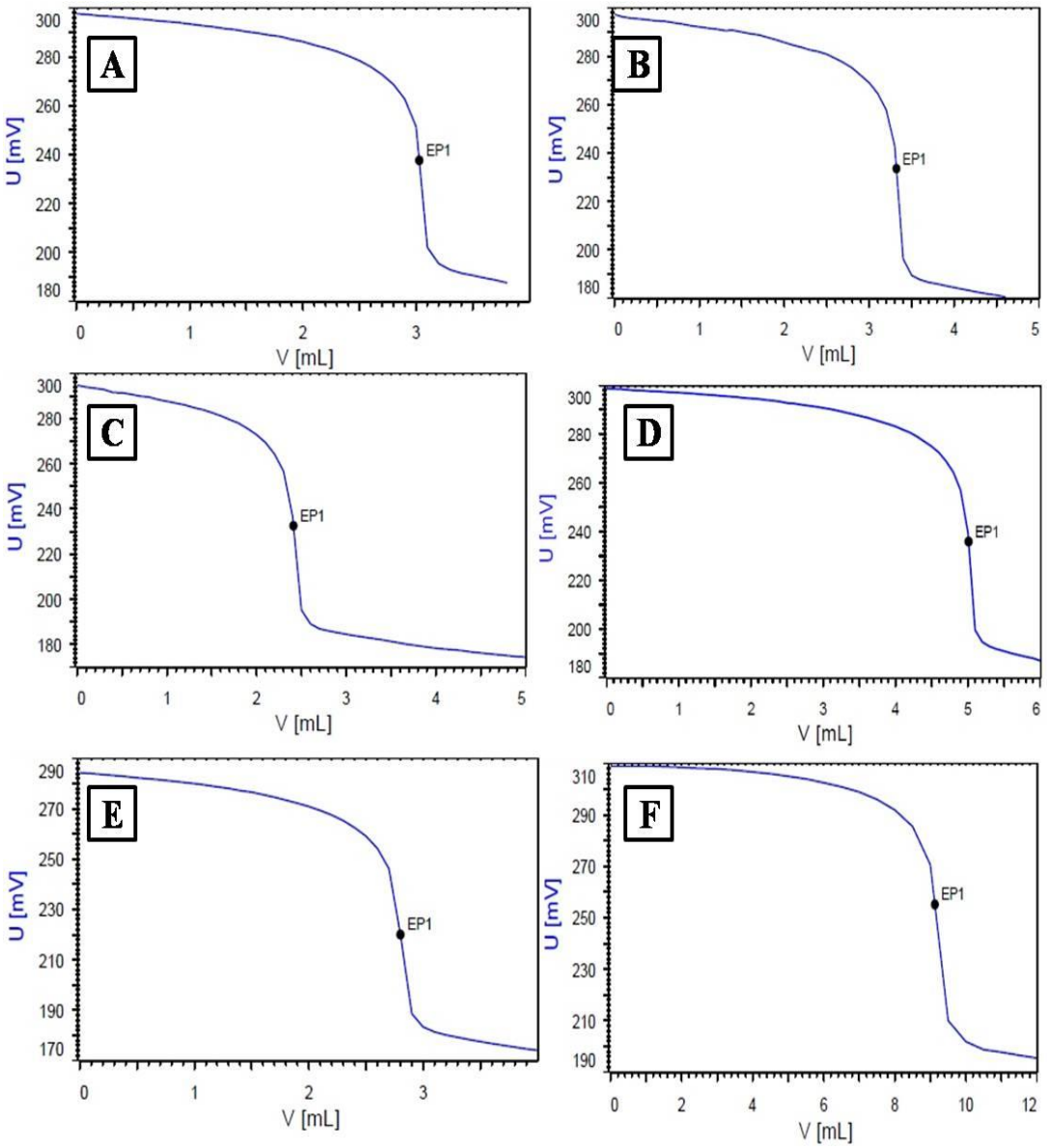


Fig. 7.11.1. Potentiometric titration curves obtained using an automatic titrator indicating the peroxide levels in; (**A**) COT_1; (**B**) COT_2; (**C**) COT_3; (**D**) COT_4; (**E**) COT_5; (**F**) COT_6

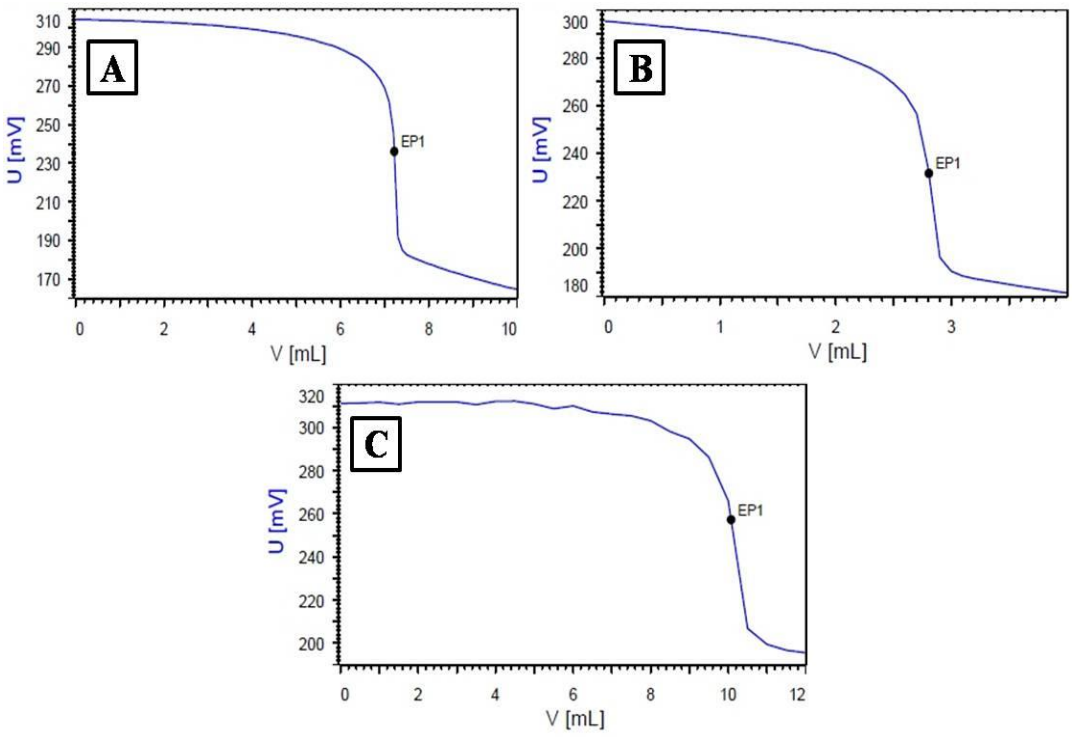


Fig. 7.11.2. Potentiometric titration curves obtained using an automatic titrator indicating the peroxide levels in; (**A**) COT_7; (**B**) COT_8 and (**C**) COT_9

The pA.V. was found to be maximum for COT_2 (34.9), followed by COT_4 (32.6) and COT_7 (32.4) after 12 days of accelerated shelf-life study. However, COT_3 could able to effectively reduced the pA.V. (30.2) after 12 days of accelerated shelf-life study (Table 7.6).

The TOTOX value was found to be maximum for COT_7 (289) followed by COT_9 (281) and COT_6 (274.4) in decreasing order. The minimum TOTOX values were observed for COT_3 (123.5) and COT_8 (134.3) (Table 7.6). The beneficiary effects of these treatments (COT_3 & COT_8) was further confirmed by its positive %SYN. These results indicated that the addition of a combination of two or three antioxidants was found to have a greater effect in reducing the deterioration rate of the concentrated sardine methyl esters than the addition of individual antioxidant.

The TBARS formation after 12 days was found to be lower in COT_3, followed by COT_8, COT_1, COT_2, COT_4, COT_6, COT_5, COT_7 and COT_9 (Table 7.6).

The DPPH scavenging activity of the CO reduced to 14.3 % from 79.4 % after 12 days (d=12) of accelerated stability study (COA, Table 7.4). The maximum

DPPH scavenging activity was observed for COT_3 (70.7 %), followed by $COT_{8,}$ $COT_{1,}$ $COT_{2,}$ $COT_{5,}$ $COT_{6,}$ $COT_{9,}$ COT_7 and COT_4 in decreasing order (Table 7.6).

Table 7.6 Characteristics of different treatments (COT_{1-9}) added with different ratios of *K. alvarezii, H. musciformis* and *J. rubens* to concentrated oil in an accelerated stability study of 12 days

		COT_1	COT_2	COT_3	COT_4	COT_5	COT_6	COT_7	COT_8	COT_9
IT		0.61	0.58	1.61	0.45	0.98	0.54	0.56	1.06	0.54
ST		0.65	0.59	3.38	0.58	1.05	0.65	0.81	1.56	0.69
AAI		4.69	4.46	12.38	3.46	7.54	4.15	4.31	8.15	4.15
%SYN		4.35	-2.17	221.74	-30.43	84.78	-10.87	-6.52	102.17	-10.87
P.V.	0	8.26±0.83^a	8.25±0.83^a	8.11±0.81^a	8.54±0.85^a	8.41±0.84^a	8.15±0.82^a	8.26±0.83^a	8.47±0.85^a	8.54±0.85^a
	4	30.31±0.03^b	48.24±0.82^b	19.23±0.92^b	21.26±0.13^b	21.46±0.15^b	21.47±0.15^b	28.06±0.81^b	22.36±0.24^b	39.46±0.95^b
	8	50.46±0.05^c	79.36±0.94^c	36.13±0.61^c	32.44±0.24^c	41.36±0.14^c	44.97±0.5^c	49.64±0.96^c	38.46±0.85^c	60.47±0.05^c
	12	60.41±0.04^d	66.36±0.64^d	46.62±0.66^d	95.99±0.6^d	53±0.03^d	121.19±0.12^d	128.41±0.84^d	51.1±0.11^d	124.63±0.46^d
pA.V.	0	10.02±0.01^a	10.25±0.03^a	9.88±0.99^a	9.89±0.9^a	10.56±0.06^a	10.25±0.03^a	10.14±0.01^a	10.35±0.04^a	10.14±0.01^a
	4	29.31±0.93^b	30.32±0.03^b	28.64±0.86^b	27.46±0.75^b	27.26±0.73^b	29.43±0.94^b	27.86±0.79^b	27.24±0.72^b	26.46±0.65^b
	8	30.21±0.02^b	33.86±0.39^b	29.61±0.96^b	30.64±0.06^b	28.63±0.86^b	31.64±0.16^b	29.21±0.92^b	29.01±0.9^b	27.64±0.76^{bc}
	12	31.25±0.13^b	34.89±0.49^b	30.23±0.02^b	32.65±0.27^b	31.42±0.14^b	32.02±0.2^b	32.45±0.25^b	32.06±0.21^b	32.01±0.2^c
TOTOX	0	26.54±0.65^a	26.75±0.68^a	26.1±0.61^a	26.97±0.7^a	27.38±0.74^a	26.55±0.66^a	26.66±0.67^a	27.29±0.73^a	27.22±0.72^a
	4	89.93±0.99^b	126.8±0.68^b	67.1±0.71^b	69.98±0.07^b	70.18±0.02^b	72.37±0.24^b	83.98±0.4^b	71.96±0.2^b	105.38±0.54^b
	8	131.13±0.11^c	192.58±0.26^c	101.87±0.19^c	95.52±0.55^c	111.35±0.14^c	121.58±0.16^c	128.49±0.85^c	105.93±0.59^c	148.58±0.86^c
	12	152.07±0.21^d	167.61±0.76^d	123.47±0.35^d	224.63±0.46^d	137.42±0.74^d	274.4±0.44^d	289.27±0.93^d	134.26±0.43^d	281.27±0.13^d
TBARS	0	0.53±0.05^a	0.51±0.05^a	0.5±0.05^a	0.46±0.05^a	0.52±0.05^a	0.54±0.05^a	0.61±0.06^a	0.59±0.06^a	0.56±0.06^a
	4	0.54±0.05^a	0.73±0.07^a	0.53±0.05^a	0.49±0.05^a	0.61±0.06^a	0.66±0.07^a	0.78±0.08^a	0.67±0.07^a	0.63±0.06^a
	8	0.71±0.07^a	0.86±0.09^a	0.61±0.06^a	0.54±0.05^a	0.89±0.09^a	0.81±0.08^a	0.91±0.09^a	0.94±0.09^a	0.71±0.07^a
	12	0.91±0.09^a	0.93±0.09^a	0.79±0.08^a	0.88±0.09^a	1.01±0.01^a	0.96±0.01^a	1.02±0.1^a	1.11±0.11^a	0.94±0.09^a
DPPH	0	96.26±0.63^a	97.41±0.74^a	99.46±0.95^a	98.67±0.87^a	98.11±0.81^a	98.01±0.8^a	97.98±0.8^a	98.26±0.83^a	98.16±0.82^a
	4	90.26±0.03^b	89.26±0.93^b	90.47±0.05^b	92.01±0.2^a	90.47±0.05^b	91.76±0.18^b	86.41±0.64^b	81.69±0.17^b	79.44±0.94^b
	8	77.21±0.72^c	78.26±0.83^c	76.36±0.64^c	74.87±0.49^b	70.64±0.06^c	73.33±0.33^c	67.69±0.77^c	66.33±0.63^c	69.46±0.95^c
	12	68.36±0.84^d	69.91±0.09^d	70.71±0.07^d	67.46±0.75^b	65.43±0.54^c	67.01±0.7^c	60.26±0.03^d	59.11±0.91^d	63.46±0.35^d

COT_{1-9} implies different treatments made by adding different combinations of *K. alvarezii*, *H. musciformis* and *J. rubens* to concentrated oil as shown in **Table 1**. IT – Induction time in h; ST – Stability time in h; AAI - Antioxidant activity index = IT with antioxidant/IT without antioxidant; The degree of synergism (% SYN) is calculated as (% SYN) = $[IT_{MIX}\text{-}IT_0]\text{-}[(IT_1\text{-}IT_0)+(IT_2\text{-}IT_0)+(IT_3\text{-}IT_0)]*100/[(IT_1\text{-}IT_0)+(IT_2\text{-}IT_0)+(IT_3\text{-}IT_0)]$., where IT_{MIX}, IT_0, IT_1, IT_2 and IT_3 are the induction periods of the samples containing the mixture of inhibitors, of the control sample, and of the samples containing the individual antioxidants. A positive value for %SYN defines a synergistic effect between the implicated antioxidants, while a negative value corresponds to an antagonistic effect. P.V. – Peroxide value represented in $meqO_2/kg$; pA.V. – p-anisidine value; TOTOX – total oxidation value (2xP.V. + pA.V.); TBARS – Thiobarbituric acid reactive species represented in mg MDA equivalent compounds/kg sample (MDAEQ/kg);DPPH – free radical scavenging activity(%).

7.2.3C. Fatty Acid Composition of Different Treatments (COT_{1-9}) Added with Different Ratios of Ethyl Acetate Fractions of K. alvarezii, H. musciformis and J. rubens to Concentrated Fatty Acid Methyl Esters in an Accelerated Stability Study of 12 Days

The fatty acid profile of the purified sardine methyl esters supplemented with antioxidative ingredients of marine origin (COT_{1-9}) to arrest the oxidation of the fatty acids under accelerated shelf storage at 0^{th} day and after 12 days are shown in Table 7.7A-B. The tertiary mixture of the marine antioxidants seemed to be more efficient on the stabilization of *n*-3 PUFA concentrates than the individual addition of each additive as evidenced nby the fatty acid profile shown in Table 7.7A-B. The fatty acid composition of COT_{1-5} and COT_{6-9} (0^{th} and 12^{th} day) during the accelerated shelf life study are shown in Table 7.7A & 7B, respectively. The percent reduction in EPA after 12^{th} day of the accelerated shelf life study compared with 0^{th} day was in the order, COT_3 (11.8 %) < COT_8 (14.8 %) < COT_2 (16.7 %) < COT_4 (17.1 %) < COT_1 (17.2 %) < COT_9 (19.2 %) < COT_7 (20.1 %) < COT_5 (20.2 %) < COT_6 (24.1 %) (Fig. 7.12). Similarly, the percent reduction in DHA after 12^{th} day of the accelerated shelf life study as compared with 0^{th} day was in the order, COT_3 (4.2 %) < COT_9 (7.2 %) < COT_5 (7.4 %) < COT_8 (8.7 %) < COT_4 (11.2 %) < COT_7 (11.4 %) < COT_1 (11.6 %) < COT_2 (15 %) < COT_6 (17.2 %). The percent reduction in ∑PUFA after 12^{th} day of the accelerated shelf life study compared with 0^{th} day was in the order, COT_3 (8.2 %) < COT_9 (9.7 %) < COT_8 (10 %) < COT_1 (11.6 %) < COT_2 (12.1 %) < COT_5 (12.3 %) < COT_4 (13.6 %) < COT_7 (20.9 %) < COT_6 (21.5 %). It is evident that the addition of natural extracts of additives with polyphenolic moieties (in COT_{1-9}) apparently shielded the sardine PUFAs against oxidation as evident by higher PUFAs in COT_{1-9}, especially, COT_3 after 12 days (d=12) of accelerated shelf life study. The percent reduction in ∑*n*-3 PUFA after 12^{th} day of the accelerated shelf life study compared with 0^{th} day was in the order., COT_3 (8.7 %) < COT_9 (12 %) < COT_8 (12.1 %) < COT_5 (14.3 %) < COT_1 (14.7 %) < COT_4 (15.1 %) < COT_2 (15.7 %) < COT_7 (17.4 %) < COT_6 (21.6 %). These results clearly indicated the beneficial effect of the treatment COT_3 as compared to other treatments (Table 7.7A&B, Fig. 7.12). The treatments

COT_3, COT_9 and COT_8 were able to effectively shield these important fatty acids from oxidative degradation until day 12 than did other treatments.

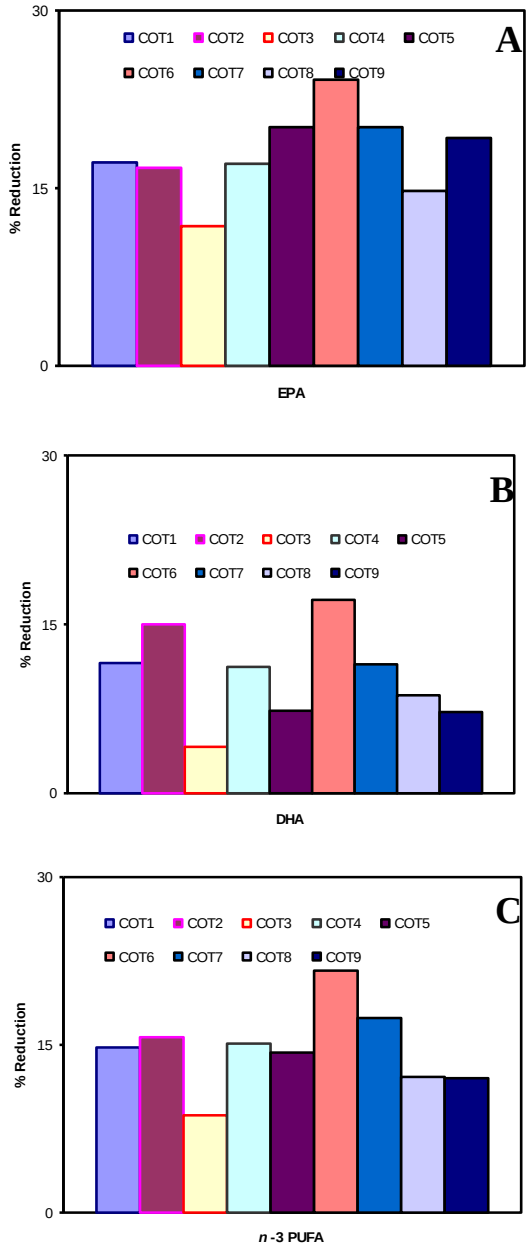


Fig.7.12 . Percent reduction in (**A**) EPA, (**B**) DHA and (**C**) ∑*n*-3PUFA after 12 days of accelerated shell life study of different treatments COT_{1-9}

Table 7.7A Fatty acid composition of COT_{1-5} at 0th and 12th day of accelerated stability life study

	COT_1		COT_2		COT_3		COT_4		COT_5	
Days	**0**	**12**	**0**	**12**	**0**	**12**	**0**	**12**	**0**	**12**
Fatty acids										
Saturated										
14:0	0.96±0.1	2.01±0.2	0.97±0.1	2.59±0.26	0.87±0.09	2.58±0.26	0.91±0.09	2.61±0.26	0.95±0.1	2.5±0.25
15:0	0.19±0.02	0.02±0	0.19±0.02	0.01±0	0.18±0.02	0.11±0.01	0.19±0.02	0.12±0.01	0.2±0.02	0.01±0
16:0	0.72±0.07	0.03±0	0.73±0.07	0.04±0	0.71±0.07	0.05±0.01	0.69±0.07	0.05±0.01	0.73±0.07	0.04±0
17:0	0.51±0.05	0.36±0.04	0.52±0.05	0.44±0.04	0.52±0.05	0.44±0.04	0.53±0.05	0.46±0.05	0.5±0.05	0.43±0.04
18:0	2.01±0.2	0.02±0*	2.11±0.21	0.03±0*	2.06±0.21	0.07±0.01*	2.1±0.21	0.02±0*	2.01±0.2	0.04±0*
20:0	0.12±0.01	0.1±0.01	0.13±0.01	0.1±0.01	0.13±0.01	0.1±0.01	0.14±0.01	0.1±0.01	0.13±0.01	0.09±0.01
22:0	0.09±0.01	0.11±0.01	0.08±0.01	0.1±0.01	0.08±0.01	0.03±0	0.09±0.01	0.1±0.01	0.14±0.01	0.05±0.01
24:0	0.13±0.01	0.14±0.01	0.13±0.01	0.15±0.02	0.11±0.01	0.12±0.01	0.11±0.01	0.12±0.01	0.16±0.02	0.1±0.01
∑SFA	4.73±0.47	2.79±0.28	4.86±0.49	3.46±0.35	4.66±0.47	3.5±0.35	4.76±0.48	3.58±0.36	4.82±0.48	3.26±0.33
Monounsaturated										
14:1*n*-7	0.11±0.01	0.11±0.01	0.1±0.01	0.12±0.01	0.09±0.01	0.12±0.01	0.09±0.01	0.11±0.01	0.1±0.01	0.1±0.01
15:1*n*-7	0.06±0.01	0.54±0.05*	0.11±0.01	0.61±0.06	0.06±0.01	0.57±0.06	0.07±0.01	0.56±0.06	0.06±0.01	0.56±0.06
16:1*n*-7 *trans*	0.02±0	0.02±0	ND	ND	0.01±0	ND	ND	ND	0.01±0	ND
16:1*n*-7	4.94±0.49	12.21±0.22*	5±0.05	13.86±0.39*	4.86±0.49	13.65±0.37*	4.87±0.49	12.85±0.29*	4.86±0.49	13.25±0.33*
18:1*n*-9 *trans*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
18:1*n*-9	5.96±0.6	5.43±0.54	4.86±0.49	5.5±0.55	5.57±0.56	5.79±0.58	5.58±0.56	5.23±0.52	5.26±0.53	5.85±0.59
20:1*n*-9	0.41±0.04	0.05±0.01	0.36±0.04	0.03±0	0.48±0.05	0.04±0	0.49±0.05	0.02±0	0.42±0.04	0.02±0
22:1*n*-9	4.26±0.43	4.16±0.42	4.11±0.41	4.7±0.47	4.31±0.43	4.85±0.49	4.3±0.43	4.61±0.46	4.31±0.43	4.58±0.46
24:1*n*-9	0.51±0.05	0.41±0.04	0.52±0.05	0.43±0.04	0.61±0.06	0.08±0.01	0.62±0.06	0.41±0.04	0.52±0.05	0.4±0.04
∑MUFA	16.27±0.63	22.93±0.29*	15.06±0.51	25.25±0.53*	15.99±0.06	25.1±0.51*	16.02±0.06	23.79±0.38*	15.54±0.55	24.76±0.48*
Polyunsaturated										
18:2*n*-6 *trans*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
18:2*n*-6	3.2±0.32	4.16±0.42	3.61±0.36	4.06±0.41	3.21±0.32	4.04±0.4	3.31±0.33	4±0.4	3.21±0.32	3.93±0.39
18:3*n*-6	0.68±0.07	1.01±0.1	0.7±0.07	1.04±0.1	0.67±0.07	1.06±0.11	0.71±0.07	1.01±0.1	0.67±0.07	0.98±0.1
18:3*n*-3	0.06±0.01	0.08±0.01	0.06±0.01	0.09±0.01	0.07±0.01	0.08±0.01	0.08±0.01	0.07±0.01	0.07±0.01	0.22±0.02
20:2*n*-6	6.02±0.6	5.26±0.53	5.01±0.5	4.95±0.5	5.96±0.6	5.05±0.51	5.87±0.59	4.01±0.4	6.11±0.61	4.87±0.49
20:3*n*-6	0.05±0.01	0.11±0.01	0.06±0.01	0.15±0.02	0.04±0	0.11±0.01	0.05±0.01	0.17±0.02	0.06±0.01	0.11±0.01
20:4*n*-6	1.11±0.11	1.26±0.13	1.08±0.11	1.19±0.12	1.11±0.11	0.19±0.02	1.01±0.1	1.21±0.12	1.01±0.1	1.11±0.11
20:5*n*-3 EPA	34.06±0.41	28.21±0.82*	32.11±0.21	26.73±0.67*	33.69±0.37	29.71±0.97	34.01±0.4	28.36±0.84*	33.96±0.04	27.1±0.71*
22:5*n*-3	2.12±0.21	1.74±0.17	2.11±0.21	1.87±0.19	2.1±0.21	1.71±0.17	2.1±0.21	1.4±0.14	2.02±0.2	1.66±0.17
22:6*n*-3 DHA	27.54±0.75	24.34±0.43	26.41±0.64	22.45±0.25	27.14±0.71	26.01±0.6	27.04±0.7	24.01±0.4	27±0.07	25.01±0.05
∑PUFA	74.84±0.48	66.17±0.62*	71.15±0.12	62.53±0.25*	73.99±0.4	67.96±0.8*	74.18±0.42	64.24±0.42*	74.11±0.41	64.99±0.5*
EPA+DHA	61.6±0.16	52.55±0.26*	58.52±0.85	49.18±0.92*	60.83±0.08	55.72±0.57*	61.05±0.11	52.37±0.24*	60.96±0.1	52.11±0.21*
∑*n*-3PUFA	63.78±0.38	54.37±0.44*	60.69±0.07	51.14±0.11*	63±0.3	57.51±0.75*	63.23±0.32	53.84±0.38*	63.05±0.31	53.99±0.4*
∑n-6PUFA	11.06±0.11	11.8±0.18	10.46±0.05	11.39±0.14	10.99±0.1	10.45±0.05	10.95±0.1	10.4±0.04	11.06±0.11	11±0.01
∑*n*-3/∑*n*-6	5.77±0.58	8.77±0.88	9.77±0.98	12.77±0.28	13.77±0.38	16.77±0.68	17.77±0.78	20.77±0.08	21.77±0.18	24.77±0.48
∑*n*-6/∑*n*-3	0.17±0.02	0.22±0.02	0.17±0.02	0.22±0.02	0.17±0.02	0.18±0.02	0.17±0.02	0.19±0.02	0.18±0.02	0.2±0.02
∑PUFA/∑SFA	15.82±0.58	23.72±0.37*	14.64±0.46	18.07±0.81	15.88±0.59	19.42±0.94*	15.58±0.56	17.94±0.79	15.38±0.54	19.94±0.09
∑TRANS	0.02±0	0.02±0	ND	ND	0.01±0	ND	ND	ND	0.01±0	ND

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. * represents a statistical difference ($p<0.05$) between 0th and 12th day. ND: not detected. COT_{1-5} is as represented in Table 7.1

Table 7.7B. Fatty acid composition of COT_{6-9} at 0^{th} and 12^{th} day of accelerated stability life study

	COT_6		COT_7		COT_8		COT_9	
Days	**0**	**12**	**0**	**12**	**0**	**12**	**0**	**12**
Fatty acids								
Saturated								
14:0	0.97±0.1	1.26±0.13	0.98±0.1	0.96±0.1	0.95±0.1	2.54±0.25	0.98±0.1	2.53±0.25
15:0	0.17±0.02	0.04±0	0.11±0.01	0.13±0.01	0.12±0.01	0.01±0	0.13±0.01	ND
16:0	0.66±0.07	0.61±0.06	0.67±0.07	0.7±0.07	0.66±0.07	0.6±0.06	0.63±0.06	0.04±0
17:0	0.48±0.05	0.55±0.06	0.49±0.05	0.61±0.06	0.44±0.04	0.43±0.04	0.43±0.04	0.42±0.04
18:0	1.67±0.17	2.63±0.26	1.67±0.17	3.64±0.36	1.62±0.16	0.05±0.01	1.63±0.16	0.09±0.01
20:0	0.11±0.01	0.15±0.02	0.16±0.02	0.26±0.03	0.17±0.02	0.09±0.01	0.18±0.02	0.1±0.01
22:0	0.09±0.01	0.09±0.01	0.08±0.01	0.08±0.01	0.09±0.01	0.08±0.01	0.09±0.01	0.03±0
24:0	0.12±0.01	0.15±0.02	0.11±0.01	0.13±0.01	0.12±0.01	0.11±0.01	0.13±0.01	0.07±0.01
∑SFA	4.27±0.43	5.48±0.55	4.27±0.43	6.51±0.65	4.17±0.42	3.91±0.39	4.2±0.42	3.28±0.33
Monounsaturated								
14:1*n*-7	0.1±0.01	0.11±0.01	0.17±0.02	0.08±0.01	0.16±0.02	0.21±0.02	0.17±0.02	0.1±0.01
15:1*n*-7	0.07±0.01	0.02±0	0.06±0.01	0.07±0.01	0.07±0.01	0.07±0.01	0.08±0.01	0.05±0.01
16:1*n*-7 *trans*	0.01±0	0.01±0	0.01±0	0.01±0	ND	ND	ND	ND
16:1*n*-7	4.65±0.47	9.36±0.94*	4.64±0.46	6.89±0.69	4.62±0.46	13.42±1.34*	4.64±0.46	13.37±1.34*
18:1*n*-9 *trans*	0.01±0	0.02±0	0.01±0	0.03±0	ND	ND	ND	ND
18:1*n*-9	5.76±0.58	6.01±0.6	5.81±0.58	11.01±1.1	5.26±0.53	5.84±0.58	5.36±0.54	5.51±0.55
20:1*n*-9	0.04±0	0.07±0.01	0.05±0.01	0.08±0.01	0.06±0.01	0.03±0	0.07±0.01	0.04±0
22:1*n*-9	4.27±0.43	5.9±0.59	4.23±0.42	8.01±0.8	4.24±0.42	4.55±0.46	4.28±0.43	4.75±0.48
24:1*n*-9	0.39±0.04	0.4±0.04	0.41±0.04	0.41±0.04	0.41±0.04	0.08±0.01	0.46±0.05	0.3±0.03
∑MUFA	15.3±0.53	21.9±0.19*	15.39±0.54	26.59±0.66*	14.82±0.48	24.2±0.42*	15.06±0.51	24.12±0.41*
Polyunsaturated								
18:2*n*-6 *trans*	ND	ND	ND	ND	ND	ND	N	ND
18:2*n*-6	3.16±0.32	3±0.3	3.11±0.31	3.11±0.31	3.12±0.31	3.98±0.4	3.23±0.32	4±0.4
18:3*n*-6	0.62±0.06	0.48±0.05	0.63±0.06	0.06±0.01	0.64±0.06	1.03±0.1	0.65±0.07	1.02±0.1
18:3*n*-3	0.05±0.01	0.01±0	0.04±0	0.01±0	0.05±0.01	0.1±0.01	0.06±0.01	0.23±0.02
20:2*n*-6	5.86±0.59	4±0.04	5.86±0.59	3.16±0.32	5.9±0.59	4.8±0.48	5.91±0.59	4.97±0.5
20:3*n*-6	0.06±0.01	0.02±0	0.07±0.01	0.01±0	0.08±0.01	0.11±0.01	0.08±0.01	0.11±0.01
20:4*n*-6	1.08±0.11	1.01±0.1	1.09±0.11	0.01±0	1.11±0.11	1.13±0.11	1.01±0.1	1.13±0.11
20:5*n*-3 EPA	33.4±3.34	25.34±0.53*	32.69±0.27	26.11±0.61*	33±0.3	28.11±0.81*	33.6±0.36	27.14±0.71*
22:5*n*-3	2.06±0.21	1.26±0.13	2.11±0.21	1.01±0.1	2.1±0.21	1.77±0.18	2.11±0.21	2.81±0.28
22:6*n*-3 DHA	27.85±0.79	23.06±0.31	27.11±0.71	24.01±0.4	27±0.7	24.65±0.47	27.6±0.76	25.6±0.06
∑PUFA	74.14±0.41	58.18±0.82*	72.71±0.27	57.49±0.75*	73±0.3	65.68±0.57*	74.25±0.43	67.01±0.7*
EPA+DHA	61.25±0.13	48.4±0.04*	59.8±0.98	50.12±0.01*	60±0.06	52.76±0.28*	61.2±0.12	52.74±0.27*
∑*n*-3PUFA	63.36±0.34	49.67±0.97*	61.95±0.2	51.14±0.11*	62.15±0.22	54.63±0.46*	63.37±0.34	55.78±0.58*
∑n-6PUFA	10.78±0.08	8.51±0.85	10.76±0.08	6.35±0.64	10.85±0.09	11.05±0.11	10.88±0.09	11.23±0.02
∑*n*-3/∑*n*-6	5.88±0.59	5.84±0.58	5.76±0.58	8.05±0.81	5.73±0.57	4.94±0.49	5.82±0.58	4.97±0.5
∑*n*-6/∑*n*-3	0.17±0.02	0.17±0.02	0.17±0.02	0.12±0.01	0.17±0.02	0.2±0.02	0.17±0.02	0.2±0.02
∑PUFA/∑SFA	17.36±0.74	10.62±0.06*	17.03±0.7	8.83±0.88*	17.51±0.75	16.8±0.68	17.68±0.77	20.43±0.04
∑TRANS	0.02±0	0.03±0	0.02±0	0.04±0	ND	ND	ND	ND

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. * represents a statistical difference ($p<0.05$) between 0^{th} and 12^{th} day. ND: not detected. COT_{6-9} is as represented in Table 7.1.

7.2.3D. Spectral analysis

The ^{1}H-NMR spectra of COA obtained after 12 days of accelerated storage (65 ^{o}C) has significantly altered with respect to its aldehydic signals (9 -10 ppm, Fig 7.13) as compared to the spectrum obtained after treatment with COT_3. The ^{1}H-NMR spectra of COA before and after accelerated storage were compared to find the variations in the proton integral values at δ 12 - 8 ppm and δ 7 – 5.8 ppm (Fig. 7.13A-C). No proton signal was noted in FAC at δ 12 - 8 ppm at the baseline (d=0) (Fig. 7.13A). However, prominent aldehydic signals (9 -10 ppm, Fig. 7.13B) in the ^{1}H-NMR spectra of COA were noted after 12 days with the aldehydic proton integral as 66. The treatment COT_3 exhibited a lesser proton integral (Fig. 7.13C; proton integral, 40.4) after 12 days. This signifies the formation of carbonyl compounds (of aldehydic nature) in the control treatment after 12th day of stability study as compared to the those with added additives. It is apparent that the oxidation of unsaturated fatty acids by oxygen, generally known as autoxidation, is important in the development of secondary reactions leading to the formation of shorter-chain carbonyl compounds mainly saturated and unsaturated aldehydes. They are important markers because, even if present in trace amounts, they impart undesirable qualities to the methyl esters. For example, the fatty acid EPA (20:5n-3) with five methylene interrupted double bonds is converted to the epoxy intermediate leading to short-lived hemiacetal, which is oxidatively cleaved to generate *cis*-3-hexenal and 13-formyltrideca-5,8,11-trienoic acid, though the positions and geometry of the double bond may change by isomerization. These short chain aldehydes generated *via* hydroperoxide intermediates are volatile compounds and have potent adverse biological effects. These results indicated that the antioxidative additives used in COT_3 could able to retain the native form of the fatty acids for a longer period on shelf resulting in the inhibition of oxidative degradation leading to the formation of low molecular weight aldehyde compounds alongwith other intermediates. The schematic diagram leading to the formation of aldehydic compounds from the precursor fatty acid (EPA) has been represented in Fig. 7.14. The addition of seaweed combination in COA also exhibited lesser proton integral at δ 7 – 5.8 ppm (33.7) as compared to COA without any additives (73.3) after the accelerated storage

study. This indicated that the seaweed based antioxidative additives could able to prevent the formation of aromatic rings and/or extended conjugation during the accelerated storage study.

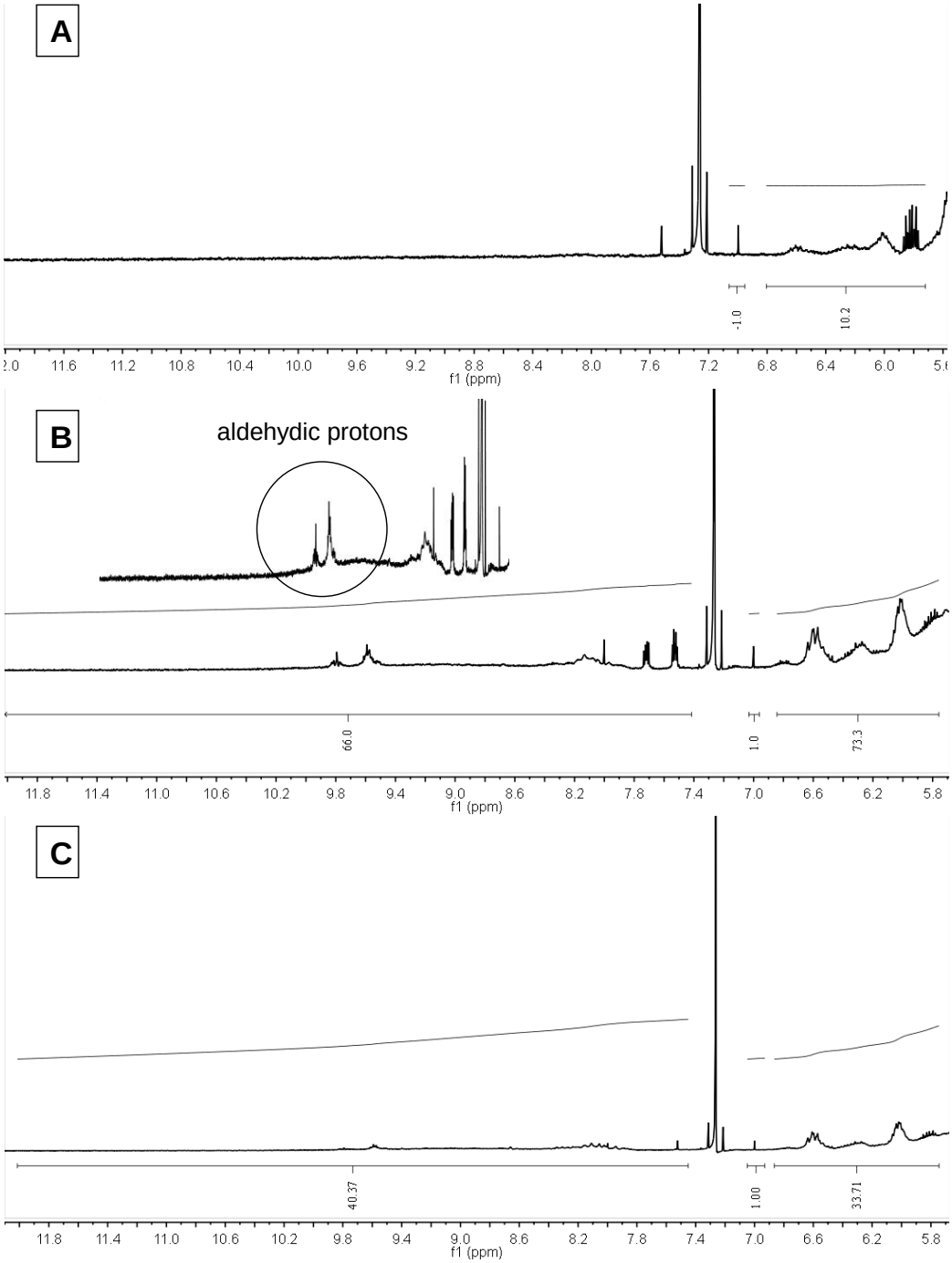


Fig. 7.13 ^{1}H-NMR spectral comparison of the concentrated fatty acid methyl esters COA at (**A**) zeroth day (**B**) 12th day and (**C**) COA added with potent treatment COT_3 after 12th day.

The spectral analysis of the concentrated fatty acid methyl esters supplemented with various combinations of additives vis-à-vis without additives was further validated using FT-IR analysis. FT-IR spectra of potent treatment COT_3 after the 12th day revealed a significant reduction in C-H stretching vibration was observed slightly higher than 3000 cm^{-1} (~ 3471 cm^{-1}), as compared to that in control, thereby explaining the absence of aromatic impurities in the concentrated fatty acid methyl esters. Compounds that do not have aromatic bonds show C-H stretches only below 3000 cm^{-1}. The intensity of the carbonyl absorption at 1741 cm^{-1} was found to be reduced in the potent treatment COT_3 after the 12th day of stability studies as compared to the control, which revealed the presence of extra carbonyl functionalities, possibly derived by the oxidation of polyunsaturated fatty acids.

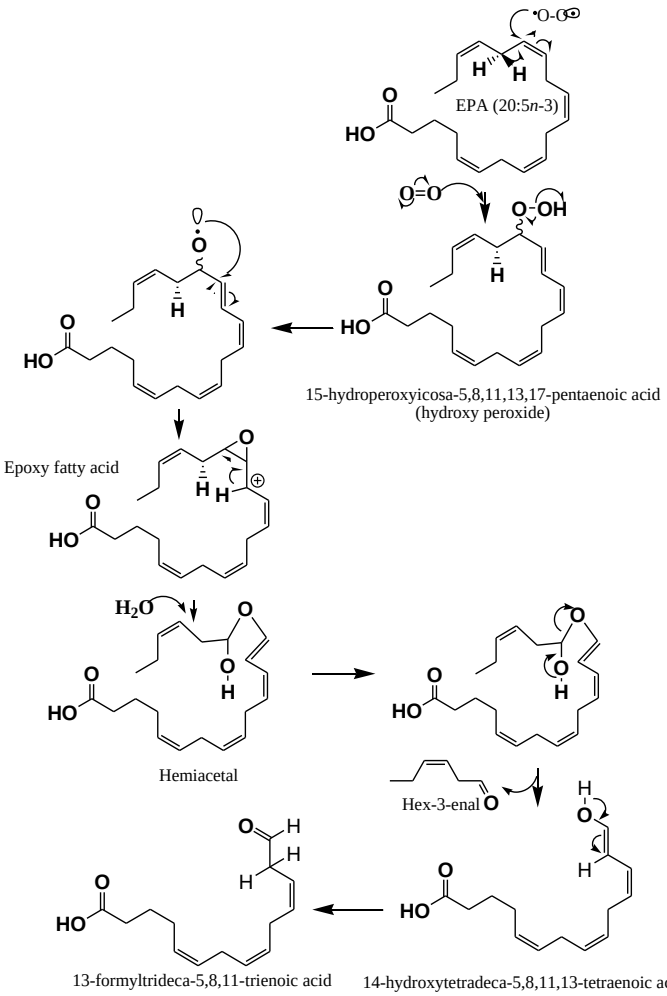


Fig. 7.14. Formation of Hex-3-enal and 13-formyltrideca-5,8,11-trienoic acid from the polyunsaturated fatty acid (EPA, 20:5*n*-3)

7.2.4. Stability Study of Purified Sardine Fatty Acid Methyl Esters (PO) Added with Different Concentrations of the Potential Antioxidant Combination (COT_3)

7.2.4A. Oil Stability Index using Rancimat Analysis

Among the different concentrations of the potential combination (COT_3), added to the purified fatty acid methyl esters (PO)., 1% (POC) and 2% (POD) concentrations showed highest IT (both 6.8 h) with higher AAI's (> 24) showing that the increase in induction period was not in proportion to concentration. Previous reports (Dziedzic & Hudson 1994) also showed that induction period increases with the concentration of primary antioxidant upto a certain optimum level., ie. the concentration at which maximum stabilizing effect is obtainable for each test antioxidant, beyond which concentration has no significant proportionate effect on stability of foods (Table 7.8) (Fig. 7.15). Dziedzic & Hudson (1994) showed that the antioxidative effects of α-tocopherol did not increase with a concentration higher than 0.05%. The present results further showed that for the low concentration of COT_3 (0.5% POB) IT was found to be significantly lower 0.41 h (AAI 1.46 h) compared to POC and POD ($p<0.05$). Tsimidou et al (1995) showed that dry oregano at 0.5% level in mackerel oil was comparable to that of 200 ppm BHA when stored at 40 °C in the dark. Similarly, the dry oregano at 1% (w/w) level had a similar effect to that of 200 ppm TBHQ.

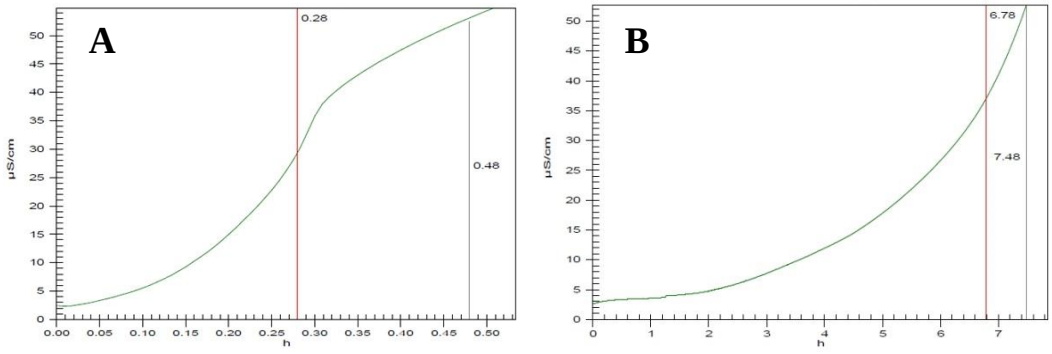


Fig. 7.15. Oil stability indices of the (**A**) purified fatty acid methyl esters POA; (**B**) purified methyl esters added with marine antioxidant blend at the ratio 1% POC

7.2.4B. P.V., pA.V., TOTOX, TBARS Values and DPPH Scavenging Activities during Accelerated Stability Study of 12 Days

P.V. after 12 days was significantly lower for 1 & 2% concentration of the potent treatment COT_3 (POC 45 $meqO_2/kg$) compared to 0, 0.5 % concentrations (266 & 79.5 $meqO_2/kg$, respectively) ($p<0.05$). At the end of the experiment (d = 12), the peroxide values of POA had the highest value of 266 $meqO_2/kg$, followed by COT_3 0.05% (POB 79.5 $meqO_2/kg$) (Fig. 7.16). POB had less significance for the retardation of autoxidation of fish methyl esters (380 % increment in P.V. after 12 days compared to 0^{th} day value) compared with POC and POD (164.1 & 213.2 % increment) ($p < 0.05$). However, the control purified methyl esters (POA) exhibited significant increment (1408 % increase) in its P.V. after 12^{th} day.

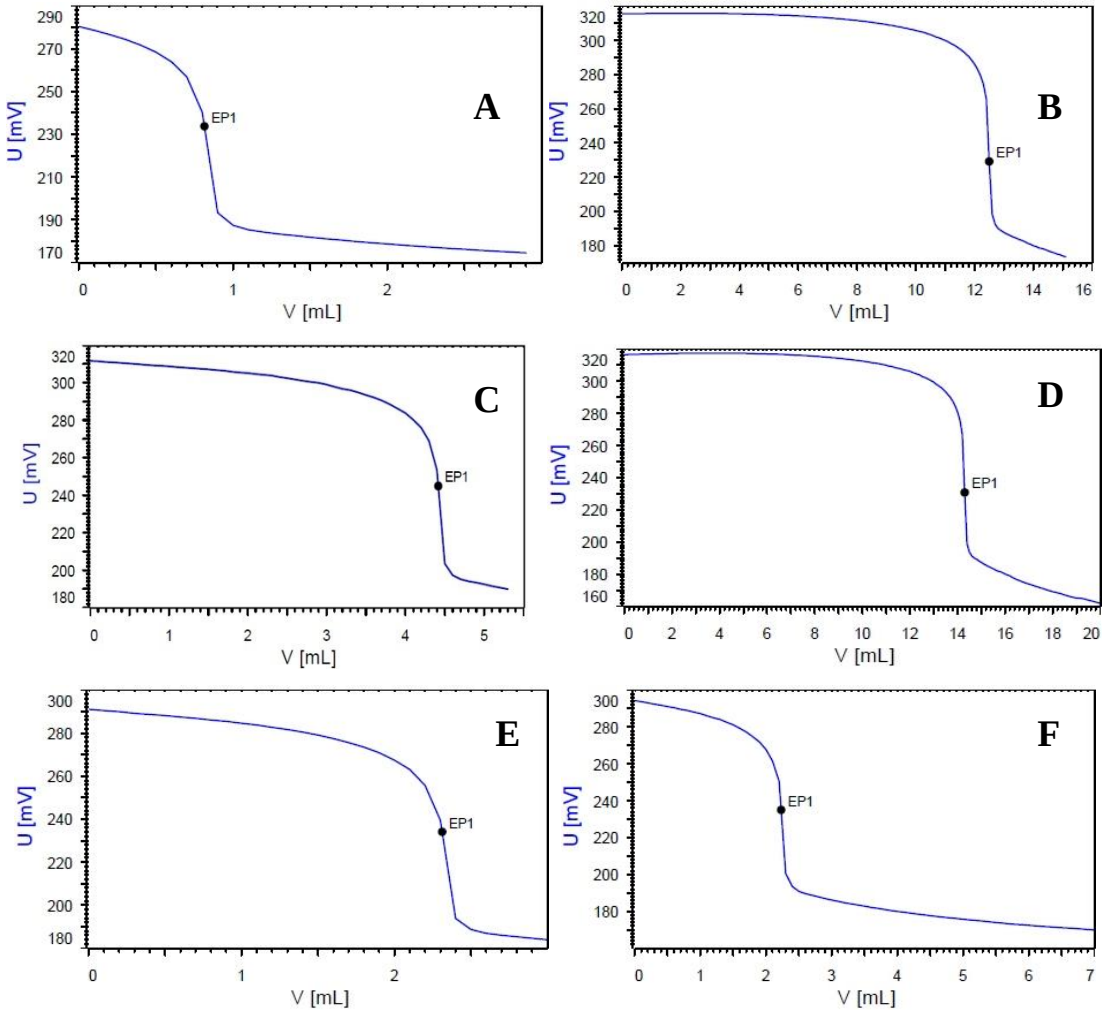


Fig. 7.16. Potentiometric titration curves obtained using an automatic titrator indicating the peroxide levels in; (**A**) POA at 0^{th} day; (**B**) POA at 8^{th} day; (**C**) POA at 12^{th} day; (**D**) POB at 12^{th} day; (**E**) POC at 12^{th} day; (**F**) POD at 12^{th} day

The pA.V. of the samples which showed 22.0 initially, increased to 36.2 at 8th day and reduced to 35.0 at 12th day for POA. On the other hand, POC and POD rose upto 30.1 and 31, respectively (d=12) (Table 7.8).

TBARS values observed were found to be elevated to 3.8 MDAEQ/kg for POA (d=12) compared to POB, POC and POD with minimum value observed for POC (~0.62 MDAEQ/kg) (Table 7.8). POC proved to be effective in protecting PO from oxidation under the stress of heating and storage,

A significantly high reduction in DPPH scavenging activity (75.8% reduction from initial value) was observed for POA, while less reduction was observed for POC followed by POD and then POB (15.6, 16.7 & 28 % reduction, respectively) (Table 7.8).

Table 7.8 Characteristics of purified methyl esters (PO) added with different concentrations (0.5, 1 and 2%) of the potent marine antioxidant combination (COT_3) in an accelerated stability study of 12 days

		POA	POB	POC	POD
IT		0.28	0.41	6.78	6.79
ST		0.48	1.05	7.48	7.45
AAI			1.46	24.21	24.25
P.V.	0	17.63±0.76^{a}	16.56±0.66^{a}	17.02±0.7^{a}	14.23±0.42^{a}
	4	84.25±0.43^{b}	25.56±0.56^{b}	19.89±0.99^{a}	15.65±0.57^{a}
	8	224.27±0.43^{c}	51.36±0.14^{c}	28.98±0.9^{b}	28.99±0.35^{b}
	12	265.93±0.59^{d}	79.52±0.95^{d}	44.95±0.94^{c}	44.57±0.46^{c}
PA.V.	0	22.02±0.2^{a}	23.69±0.37^{a}	24.01±0.4^{a}	23.22±0.32^{a}
	4	29.01±0.9^{b}	26.45±0.65^{ab}	26.78±0.68^{ab}	25.43±0.54^{a}
	8	36.23±0.62^{c}	28.25±0.83^{ab}	29.88±0.99^{ab}	29.23±0.92^{ab}
	12	35.01±0.5^{c}	31.4±0.14^{b}	30.1±0.01^{b}	31.01±0.1^{b}
TOTOX	0	57.28±0.73^{a}	56.81±0.68^{a}	58.05±0.81^{a}	51.68±0.17^{a}
	4	197.51±0.75^{b}	77.57±0.76^{b}	66.56±0.66^{a}	56.73±0.67^{a}
	8	484.77±0.48^{c}	130.97±0.1^{c}	87.84±0.78^{b}	96.31±0.63^{b}
	12	566.87±0.69^{d}	190.44±0.04^{d}	108.96±0.9^{c}	120.15±0.02^{b}
TBARS	0	1.54±0.15^{a}	0.56±0.06^{a}	0.51±0.05^{a}	0.56±0.06^{a}
	4	2.05±0.21^{a}	0.62±0.06^{a}	0.58±0.06^{a}	0.58±0.06^{a}
	8	3.06±0.31^{a}	0.6±0.06^{a}	0.61±0.06^{a}	0.61±0.06^{a}
	12	3.83±0.38^{a}	0.71±0.06^{a}	0.62±0.06^{a}	0.64±0.06^{a}
DPPH	0	96.01±0.6^{a}	99.26±0.93^{a}	99.55±0.96^{a}	99.64±0.96^{a}
	4	96.05±0.61^{a}	93.46±0.35^{b}	92.36±0.24^{b}	93.05±0.31^{bc}
	8	55.05±0.51^{b}	87.45±0.75^{c}	89.33±0.93^{b}	89.22±0.92^{c}
	12	23.21±0.32^{c}	71.44±0.14^{d}	83.98±0.04^{c}	83.01±0.3^{d}

POA = PO - Purified methyl esters obtained by Ag-silica chromatography (Chapter - 5); POB – POA + 0.5% COT_3 , POC - POA + 1% COT_3, POD - POA + 2% COT_3. IT – Induction time in h; ST – Stability time in h; P.V. – Peroxide value represented in meqO_2/kg; pA.V. – p-anisidine value; TOTOX – total oxidation value (2 x P.V. + pA.V.); TBARS – Thiobarbituric acid reactive species represented in mg MDA equivalent compounds/kg sample (MDAEQ/kg); DPPH – free radical scavenging activity(%).

7.2.4C. Fatty Acid Composition during Accelerated Shelf Life Study of 12 Days

The fatty acid composition of the purified oil POA (the fraction FS_6; *Chapter 5*) during accelerated stability study is shown in Table 7.9A. The SFAs in POA accounted for 5.5 % at 12th day compared to the initial value of 0.3 % ($p<0.05$). However, ∑SFA for POC and POD increased to only 2.9 % at 12th day. The MUFA content was found to be increased to 20 % from the initial value of 1.8 %.. ∑PUFA for POA showed high percent reduction after 12 days of accelerated shelf life study. ∑PUFA reduced to 82.6 % in the 4th day from the initial value of 97.3 %. EPA showed 70 % reduction whereas DHA showed 36.4 % reduction from its initial day value. The percent reduction in EPA was significantly lower in both POC and POD (< 39%). However, the marine antioxidant combination added to POA at 1% showed significantly lower reduction (54 %) compared to POC and POD ($p<0.05$). ∑*n*-3 PUFA (87.8 %) reduced to 70.5 % in the 4th day, 60 % in the 8th day and 46.5 % in the 12th day for POA (% reduction compared to d=0; 47.1%). A lower reduction in DHA content was observed for POA (31 % reduction), followed by POC, POB and POA (34.1, 34.3, 36.2 %, respectively) (Table 7.9B). The similar shelf-life pattern of POC (1%) and POD (2%) in degradation of PUFAs can be explained by the fact that a threshold level of the antioxidant combination COT_3 (1 % of the blend KAF + HMF + JRF, 0.1:0.2:0.2) is sufficient for optimum antioxidant activity, and further addition of antioxidants (as 2 % in POD) does have negative impact on stability of the fatty acid.

The accelerated shelf life study showed the isomerization of naturally present *cis* double bonds in PUFAs to their *trans* conformers when no additives were used. Also of note is that the formation of ∑*trans* fatty acids (d=12) were significantly higher ($p<0.05$) in POA (18.0 %) with respect to initial value (d=0; 0.09 %) than in the treatments (POB, POC, POD) added with marine antioxidant combination (COT_3). POC and POD realized a decelerated formation of *trans* fatty acids apparently due to the presence of marine antioxidant combination (COT_3). The 20:5*n*-3 *trans* exhibited a significant increase (from 0 to 7.6 %) in POA after accelerated shelf life study of 12 days. The combinations, POC even with less percent of additive concentration (1%) were found to be efficient to completely arrest the isomeric conversion of 18:1*n*-9 and 18:2*n*-6 *cis* to their *trans* conformers at the end accelerated shelf life study (d=12). The *trans* 18:2*n*-6 was observed to be increased to 0.19 % from 0.09 %. These increase of *trans* isomers of fatty acids is related to the initiation of lipid peroxidation, which is initiated by a homogeneous

split of a C-H bonf adjacent to *cis* double bonds in unsaturated fatty acids. A mechanism showing the free radical mediated modification of 9c, 12c-18:2*n*-6 to the *trans* geometrical isomers 9t, 12c-18:2*n*-6, 9c, 12t-18:2*n*-6, and 9t, 12t-18:2*n*-6 is shown in Fig. 7.18.

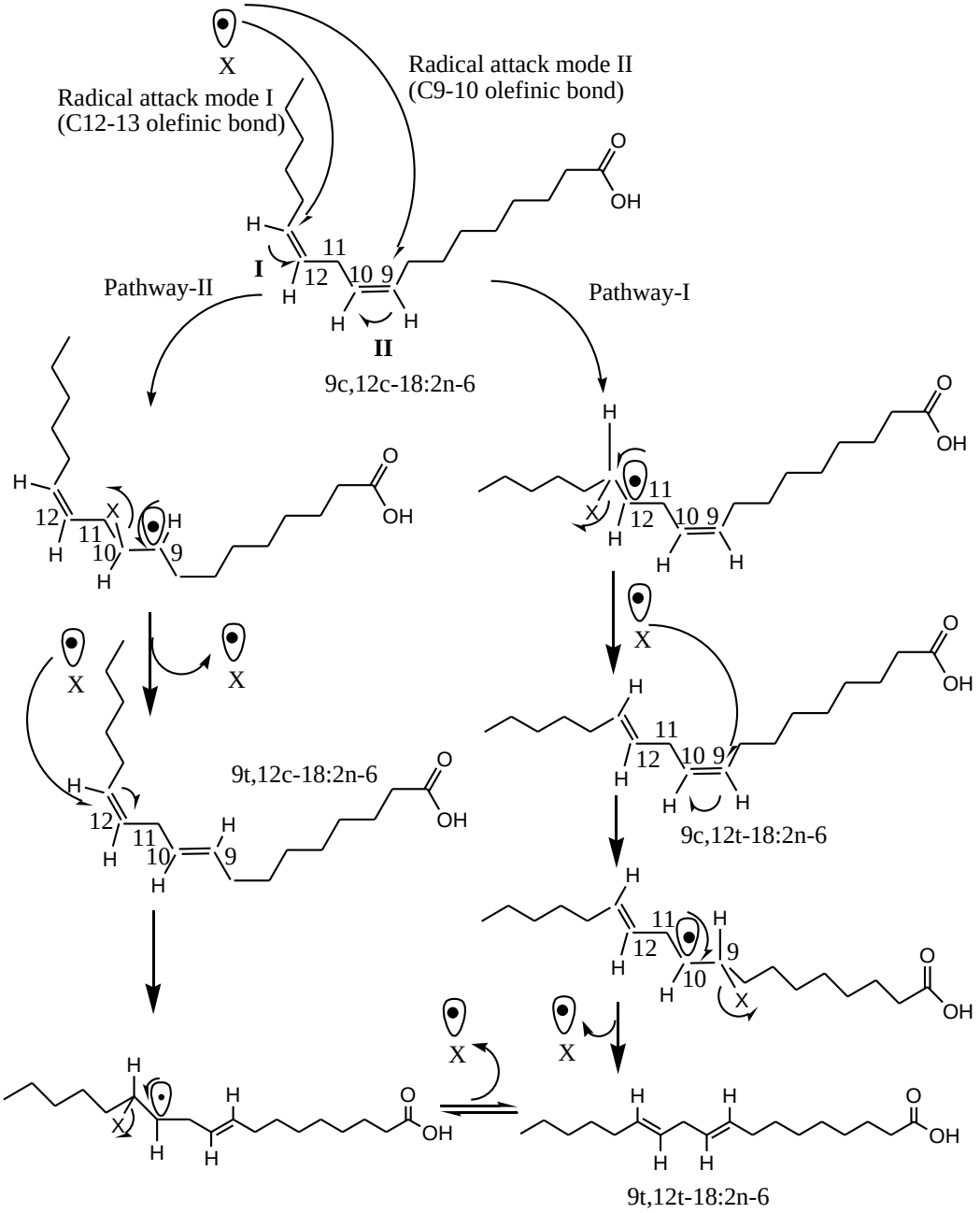


Fig. 7.17. The free radical mediated modification of 9c, 12c-18:2*n*-6 to the *trans* geometrical isomers 9t, 12c-18:2*n*-6, 9c, 12t-18:2*n*-6, and 9t, 12t-18:2*n*-6.

Table 7.9A Fatty acid composition of POA during accelerated stability life study of 12 days

Fatty acids	POA			
	0	4	8	12
Saturated				
14:0	0.01±0	0.06±0.01	1.01±0.1*	1.88±0.19*
15:0	ND	0.1±0.01	0.26±0.03	0.39±0.04
16:0	ND	0.01±0	0.36±0.04	1.21±0.12
17:0	0.01±0	0.04±0	0.32±0.03	0.56±0.06
18:0	0.01±0	0.11±0.01	0.16±0.02	0.34±0.03
20:0	0.04±0	0.08±0.01	0.13±0.01	0.43±0.04
22:0	0.04±0	0.01±0	0.04±0	0.14±0.01
24:0	0.19±0.02	0.21±0.02	0.23±0.02	0.58±0.06
∑SFA	0.3±0.03	0.62±0.06	2.51±0.25*	5.53±0.55*
Monounsaturated				
14:1*n*-7	0.01±0	0.16±0.02	0.24±0.02	0.58±0.06
15:1*n*-7	0.01±0	0.01±0	0.01±0	0.1±0.01
16:1*n*-7 *trans*	ND	0.16±0.02	0.21±0.02	0.31±0.03
16:1*n*-7	0.43±0.04	1.2±0.12	8.34±0.83*	14.12±1.41*
18:1*n*-9 *trans*	ND	0.01±0	0.08±0.01	0.12±0.01
18:1*n*-9	0.58±0.06	0.99±0.1	2.01±0.2	3.21±0.32
20:1*n*-9	0.03±0	0.06±0.01	0.09±0.01	0.12±0.01
22:1*n*-9	0.34±0.03	0.36±0.04	0.91±0.09	1.18±0.12
24:1*n*-9	0.43±0.04	0.53±0.05	0.32±0.03	0.22±0.02
∑MUFA	1.83±0.18	3.48±0.35	12.21±1.22*	19.96±2*
Polyunsaturated				
18:2*n*-6 *trans*	ND	0.06±0.01	0.12±0.01	0.24±0.02
18:2*n*-6 *cis*	5.28±0.53	4.06±0.41	3.86±0.39	3.09±0.31
18:3*n*-6 *trans*	0.09±0.01	0.78±0.08	0.04±0	0.19±0.02
18:3*n*-6 *cis*	0.65±0.07	0.78±0.08	0.64±0.06	0.46±0.05
18:3*n*-3	0.03±0	0.06±0.01	0.09±0.01	0.12±0.01
20-2*n*-6	3.06±0.31	2.01±0.2	1.86±0.19	1.47±0.15
20:3*n*-6	0.1±0.01	0.01±0	0.23±0.02	0.17±0.02
20:4*n*-6	0.37±0.04	0.38±0.04	0.38±0.04	0.39±0.04
20:5*n*-3 *trans*	ND	3.26±0.33	5.64±0.56*	7.58±0.76*
20:5*n*-3 *cis*	47.55±4.76	35.46±3.55*	26.21±2.62*	14.24±1.42*
22:5*n*-3 *trans*	ND	0.26±0.03	4.81±0.48	8.64±0.86
22:5*n*-3 *cis*	2.05±0.21	4.86±0.49	5.02±0.5	7.77±0.78*
22:6*n*-3 *trans*	ND	0.41±0.04	0.96±0.1	1.06±0.11
22:6*n*-3 *cis*	38.15±3.82	30.16±3.02*	28.71±2.87*	24.33±2.43*
∑PUFA	97.33±9.73	82.55±8.26*	78.57±7.86*	69.75±6.98*
EPA+DHA	85.7±8.57	65.62±6.56*	54.92±5.49*	38.57±3.86*
∑*n*-3PUFA	87.78±8.78	70.54±7.05*	60.03±6*	46.46±4.65*
∑*n*-6PUFA	8.9±0.89	7.24±0.72	6.37±0.64	5.31±0.53
∑*n*-3/∑n-6	9.86±0.99	9.74±0.97	9.42±0.94	8.75±0.88
∑*n*-6/∑*n*-3	0.1±0.01	0.1±0.01	0.11±0.01	0.11±0.01
∑PUFA/∑SFA	324.43±32.44	133.15±13.32*	31.3±3.13*	12.61±1.26*
∑TRANS	0.09±0.01	4.94±0.49*	11.86±1.19*	18.14±1.81*

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. * represents a statistical difference ($p<0.05$) in the value compared to 0^{th} day. ND: not detected. POA = PO - Purified methyl esters obtained by Ag-silica chromatography (*Chapter 5*)

Table 7.9B Fatty acid composition of POB-POD during accelerated stability study of 12 days

Fatty acids	POB				POC				POD			
	0	4	8	12	0	4	8	12	0	4	8	12
Saturated												
14:00	0.08±0.01	0.26±0.03	0.54±0.05	1.96±0.2*	0.09±0.01	0.06±0.01	0.05±0.01	0.03±0	0.09±0.01	0.06±0.01	0.05±0.01	0.02±0
15:00	0.02±0	0.08±0.01	0.16±0.02	0.46±0.05*	0.02±0	0.05±0.01	0.11±0.01	0.14±0.01	0.03±0	0.04±0	0.11±0.01	0.15±0.02
16:00	0.02±0	0.16±0.02	0.41±0.04	0.97±0.1*	0.01±0	0.11±0.01	0.26±0.03	0.68±0.07*	0.01±0	0.11±0.01	0.27±0.03	0.67±0.07
17:00	0.06±0.01	0.14±0.01	0.31±0.03	0.44±0.04	0.01±0	0.05±0.01	0.55±0.06	0.84±0.08*	0.02±0	0.05±0.01	0.55±0.06	0.85±0.09
18:00	0.09±0.01	0.1±0.01	0.16±0.02	0.31±0.03	0.01±0	0.02±0	0.21±0.02	0.34±0.03	0.01±0	0.01±0	0.24±0.02	0.35±0.04
20:00	0.06±0.01	0.09±0.01	0.14±0.01	0.38±0.04	0.04±0	0.05±0.01	0.21±0.02	0.34±0.03	0.03±0	0.05±0.01	0.21±0.02	0.34±0.03
22:00	0.05±0.01	0.11±0.01	0.16±0.02	0.21±0.02	0.04±0	0.06±0.01	0.25±0.03	0.39±0.04	0.05±0.01	0.06±0.01	0.26±0.03	0.39±0.04
24:00:00	0.11±0.01	0.04±0	0.31±0.03	0.36±0.04	0.19±0.02	0.21±0.02	0.12±0.01	0.11±0.01	0.18±0.02	0.22±0.02	0.12±0.01	0.16±0.02
∑SFA	0.49±0.05	0.98±0.1	2.19±0.22	5.09±0.51	0.41±0.04*	0.61±0.06	1.76±0.18	2.87±0.29	0.42±0.04	0.6±0.06	1.81±0.18	2.93±0.29
Monounsaturated												
14:1 *n-7*	0.02±0	0.16±0.02	0.24±0.02	0.36±0.04	0.16±0.02	0.21±0.02	1.2±0.12	2.84±0.28	0.17±0.02	0.21±0.02	1.02±0.1	2.86±0.29
15:1 *n-7*	0.01±0	0.02±0	0.04±0	0.08±0.01	0.02±0	0.01±0	0.05±0.01	0.08±0.01	0.02±0	0.01±0	0.06±0.01	0.07±0.01
16:1 *n-7 trans*	ND	0.11±0.01	0.24±0.02	0.32±0.03	0.01±0	0.03±0	0.04±0	0.08±0.01	0.01±0	0.03±0	0.04±0	0.08±0.01
16:1 *n-7*	0.44±0.04	2.05±0.21	8.06±0.81*	11.65±1.17*	0.36±0.04	1.02±0.1	3.25±0.33	6.56±0.66	0.32±0.03	1.01±0.1	3.01±0.3	6.88±0.69
18:1 *n-9 trans*	ND	0.01±0	0.08±0.01	0.09±0.01	ND	0.01±0	0.04±0	0.08±0.01	ND	0.01±0	0.04±0	0.09±0.01
18:1 *n-9*	0.56±0.06	1.04±0.1	2.01±0.2	3.41±0.34	0.58±0.06	0.89±0.09	2.01±0.2	4.36±0.44*	0.57±0.06	0.87±0.09	2.11±0.21	4.24±0.42
20:1 *n-9*	0.03±0	0.06±0.01	0.14±0.01	0.26±0.03	0.03±0	0.04±0	0.35±0.04	0.53±0.05	0.03±0	0.04±0	0.35±0.04	0.54±0.05
22:1 *n-9*	0.36±0.04	0.37±0.04	0.33±0.03	1.96±0.2	0.34±0.03	0.69±0.07	1.56±0.16	2.34±0.23	0.32±0.03	0.68±0.07	1.65±0.17	2.26±0.23
24:1 *n-9*	0.44±0.04	0.36±0.04	0.31±0.03	0.24±0.02	0.42±0.04	0.3±0.03	0.21±0.02	0.28±0.03	0.41±0.04	0.02±0	0.21±0.02	0.29±0.03
∑MUFA	1.86±0.19	4.18±0.42	11.45±1.15*	18.37±1.84*	1.92±0.19	3.2±0.32	8.71±0.87*	17.15±1.72*	1.85±0.19	2.88±0.29	8.49±0.85*	17.31±1.73*
Polyunsaturated												
18:2 *n-6 trans*	0.26±0.03	0.16±0.02	0.14±0.01	0.13±0.01	ND	ND	0.02±0	0.03±0	ND	4.8±0.48	0.01±0	0.06±0.01
18:2 *n-6 cis*	3.01±0.3	4.01±0.4	3.87±0.39	3.64±0.36	3.31±0.33	5.2±0.52	4.98±0.5	4.95±0.5	5.29±0.53	5.2±0.52	4.25±0.43	4.94±0.49
18:3 *n-6 trans*	0.08±0.01	0.1±0.01	0.16±0.02	0.21±0.02	ND	ND	ND	ND	ND	ND	ND	ND
18:3 *n-6 cis*	0.86±0.09	0.86±0.09	0.56±0.06	0.48±0.05	0.9±0.09	0.56±0.06	0.35±0.04	0.2±0.02	0.48±0.05	0.46±0.05	0.34±0.03	0.16±0.02
18:3 *n-3*	0.04±0	0.08±0.01	0.09±0.01	0.13±0.01	0.03±0	0.09±0.01	0.1±0.01	0.11±0.01	0.03±0	0.08±0.01	0.65±0.07	0.12±0.01
20-2 *n-6*	3.05±0.31	2.84±0.28	2.01±0.2	1.82±0.18	3.08±0.31	3.21±0.32	3.68±0.37	3.91±0.39	3.11±0.31	3.02±0.3	3.69±0.37	3.86±0.39
20:3 *n-6*	0.11±0.01	0.16±0.02	0.17±0.02	0.21±0.02	0.1±0.01	0.15±0.02	0.16±0.02	0.23±0.02	0.1±0.01	0.15±0.02	0.16±0.02	0.16±0.02
20:4 *n-6*	0.39±0.04	0.43±0.04	0.61±0.06	0.77±0.08	0.38±0.04	0.45±0.05	0.65±0.07	0.79±0.08	0.38±0.04	0.45±0.05	0.65±0.07	0.77±0.08
20:5 *n-3 trans*	ND	0.06±0.01	0.84±0.08	1.02±0.1*	ND	0.05±0.01	0.08±0.01	0.11±0.01	ND	0.05±0.01	0.08±0.01	0.09±0.01
20:5 *n-3 cis*	47.88±4.79	38.01±3.8*	31.02±3.1*	22.06±2.21*	47.89±4.79	42.69±4.27	39.02±3.9*	29.42±2.94*	47.56±4.76	42.03±4.2	39.69±3.97*	29.96±3*
22:5 *n-3 trans*	0.01±0	0.16±0.02	3.89±0.39	8.02±0.8	ND	0.05±0.01	0.09±0.01	0.14±0.01	ND	0.05±0.01	0.09±0.01	ND
22:5 *n-3 cis*	2.06±0.21	5.01±0.5	7.24±0.72*	8±0.8*	2.06±0.21	3.02±0.3	4.56±0.46	8.56±0.86*	2.01±0.2	3±0.3	4.65±0.47	6.05±0.61
22:6 *n-3 trans*	ND	0.06±0.01	0.21±0.02	0.42±0.04	ND	0.05±0.01	0.06±0.01	0.1±0.01	ND	0.05±0.01	0.06±0.01	0.11±0.01
22:6 *n-3 cis*	38.06±3.81	32.66±3.27*	31.96±3.2*	25±2.5*	38.27±3.83	34.21±3.42	30.65±3.07*	25.2±2.52*	38.01±3.8	33.69±3.37	30.99±3.1*	26.21±2.62*
∑PUFA	95.81±9.58	84.6±8.46*	82.77±8.28*	71.91±7.19*	96.02±9.6	89.73±8.97	84.4±8.44	73.75±7.38	96.97±9.7	93.03±9.3	85.31±8.53*	72.49±7.25*
EPA+DHA	85.94±8.59	70.67±7.07*	62.98±6.3*	47.06±4.71*	86.16±8.62	76.9±7.69	69.67±6.97	54.62±5.46	85.57±8.56	75.72±7.57	70.68±7.07*	56.17±5.62*
∑*n-3*PUFA	88.04±8.8	75.76±7.58*	70.31±7.03*	55.19±5.52*	88.25±8.83	80.01±8	74.33±7.43	63.29±6.33	87.61±8.76	78.8±7.88	75.98±7.6*	62.34±6.23*
∑*n-6*PUFA	6.64±0.66	7.54±0.75	6.82±0.68	6.65±0.67	6.87±0.69	9.01±0.9	9.47±0.95	9.88±0.99	8.88±0.89	8.82±0.88	8.75±0.88	9.73±0.97
∑*n-3*/∑n-6	13.26±1.33	10.05±1.01	10.31±1.03	8.3±0.83*	12.85±1.29	8.88±0.89	7.85±0.79	6.41±0.64	9.87±0.99	8.93±0.89	8.68±0.87	6.41±0.64
∑*n-6*/∑*n-3*	0.08±0.01	0.1±0.01	0.1±0.01	0.12±0.01	0.08±0.01	0.11±0.01	0.13±0.01	0.16±0.02	0.1±0.01	0.11±0.01	0.12±0.01	0.16±0.02
∑PUFA/∑SFA	195.53±19.5	86.33±8.63*	37.79±3.78*	14.13±1.41*	234.2±23.42	147.1±14.71	47.95±4.8	25.7±2.57	230.88±23.09	155.05±15.51*	47.13±4.71*	24.74±2.47*
∑TRANS	0.35±0.04	0.66±0.07	5.56±0.56*	10.21±1.02*	0.01±0	0.19±0.02	0.33±0.03	0.54±0.05	0.01±0	4.99±0.5	0.32±0.03	0.43±0.04

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. * represents a statistical difference ($p<0.05$) between 0^{th} and 12^{th} day. ND: not detected.); POB – POA + 0.5% COT_3 , POC - POA + 1% COT_3,

7.2.4D. Spectral Analysis of the Purified Fatty Acid Methyl Esters (PO) Added with Marine Extract Combination (POC, 1%) Compared with PO without Additives (POA) in an Accelerated Stability Study of 12 Days

^{1}H-NMR in the present study demonstrated the non-selective lipid oxidation showing specific primary and secondary lipid oxidation products along with other oxidative changes during the 12 days of accelerated storage. The proton integral in ^{1}H-NMR spectra of purified fatty acid methyl esters (POA) before and after accelerated storage period along with PO added to the potent seaweed extract combination (at 1 %, POC) was calculated (Fig. 7.18A-C). The proton integral demonstrated the increase in the characteristic proton signals at δ 7.5 – 8 ppm in POA to about 168.3 after day 12 from the baseline proton integral of 6.8, which showed that there were increased formations of secondary oxidations products belonging to aromatic groups. A chain of transformations leading to the formation of aromatic ring hypothetically consists of different stages consisting of isomerization of polyenes and double bond shifting to afford thermodynamically stable conjugated polyenes. The process of aromatic ring formation might possibly occur in the process of cyclization of the adjoint polyene to cyclohexadiene with the unsaturated hydrocarbon radical followed by dehydrogenation of the cyclohexadiene ring to the aryl ring. It is significant to note that the purified fatty acid methyl esters added with the seaweed extract combination (POC) did not show any prominent signal in this region (proton integral of ~ 16.4) apparently due to the stabilizing effect of the antioxidative compounds.

An increase in the proton integral at about δ5.4 ppm (~ 14280) was apparent in the ^{1}H NMR of the purified fatty acid methyl esters (control POA) as compared to the baseline value (~ 122.4), which apparently indicated the formation of additional olefinic bonds in the control (POA), preferably of conjugated nature after 12 days of stability study. The purified fatty acid methyl esters added with seaweed antioxidants arrested the significant increase in the formation of conjugated olefinic double bond as demonstrated by the lesser olefinic proton integral (~950) after the accelerated storage study. The configuration of the double bond protons was

determined by the coupling constant (*J* values) of olefinic protons, and the lesser *J* value signified the presence of *cis* double bond. The -CH_2- protons adjacent to the *cis* double bonds as in POC added with seaweed extract combination before and after 12 days of the accelerated stability study exhibited a resonance at about 2.5 ppm, which were sharper (less than 2 ppm) than that produced by a *trans* double bond in control POA after the study period. This demonstrated that there was increased formation of *trans* isomers of fatty acids in the control over the study period of 12 days, where no antioxidative additives have been added. The results also support the information obtained from the fatty acid profile. The distinct increase in the proton integral at about δ 4-4.3 ppm in the ^{1}H NMR of the control (~ 4229) than those obtained at baseline (~7.9) apparently demonstrated the formation of the additional secondary oxidation products, and was assigned to the presence of -CH_2- and/or isopropyl groups linked to the electronegative ester or acid groups. The lesser proton integral (~ 59) of the POC (added with seaweed antioxidants) indicated the that these marine derived antioxidative additives could able to arrest the formation the additional secondary oxidation products characterized at δ 4-4.3 ppm region of the ^{1}H NMR spectrum.

A chain of transformations leading to the formation of aromatic ring hypothetically consists of different stages consisting of isomerization of polyenes and double bond shifting to afford thermodynamically stable conjugated polyene (for example, icosa-1,3,5,7,9-pentaene derivative from the fatty acid EPA). Different adjoint polyenes are capable of the coordinated cyclization under the influence of oxidative factors. The process of aromatic ring formation may possible takes place by the process of cyclization of the adjoint polyene to cyclohexadiene with the unsaturated hydrocarbon radical followed by dehydrogenation of the cyclohexadiene ring to the aryl ring. Further aromatization reaction may lead to the formation of the naphthalene and phenanthrene ring (depending upon the number of double bonds) as shown in the following diagram (Fig. 7.19).

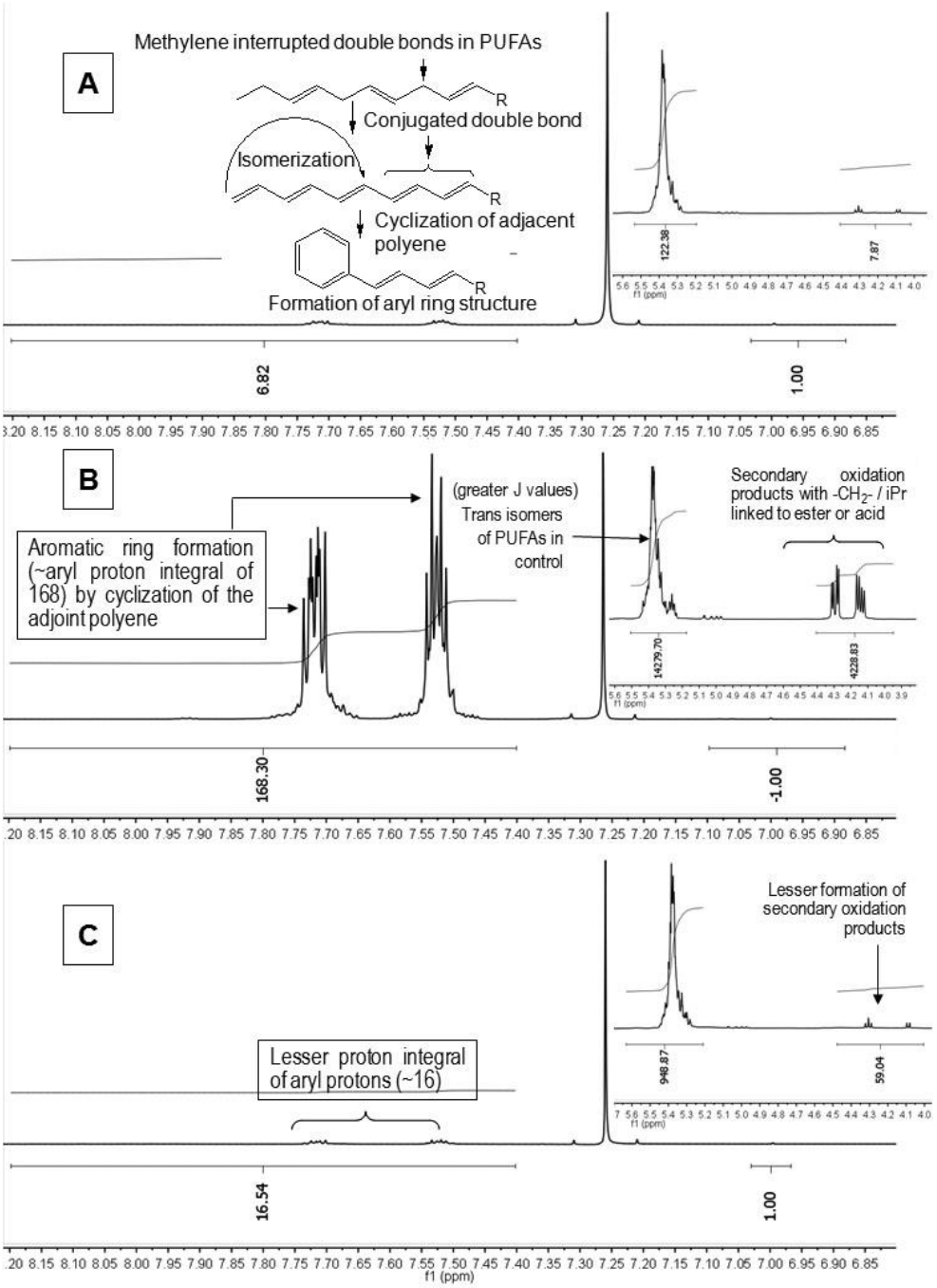


Fig. 7.18. ^{1}H-NMR spectral comparison of (**A**) purified fatty acid methyl esters of the *n*-3 PUFA concentrate derived by argentated chromatography (POA) at baseline (d=0). The chain of transformations leading to the formation of aromatic ring from PUFAs was shown as inset; (**B**) POA after accelerated storage (d=12). The enlargement of signals at δ 7-8 ppm and δ4 – 5.5 ppm were shown. The arrows indicate the regions showing the main changes due to the formation of secondary oxidation products and aromatic ring system; (**C**) PO added with

seaweed combination *K. alvarezii: H. musciformis: J. rubens* at 0.1:0.2:0.2 ratio (%, w/w), POC, after accelerated storage period together with the enlargement of several signals. No new resonances appeared due to the formation of aromatic rings and additional secondary oxidation products.

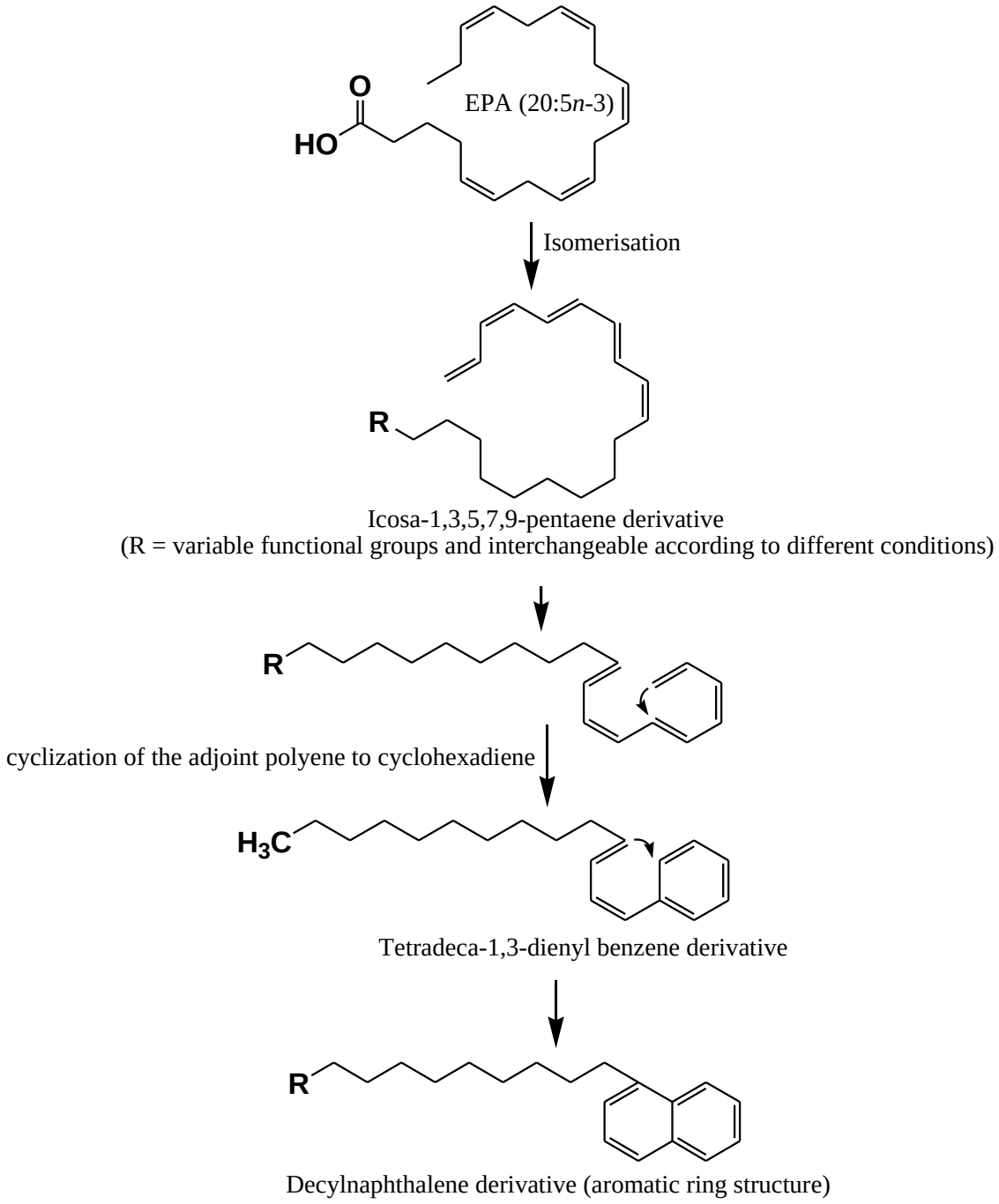
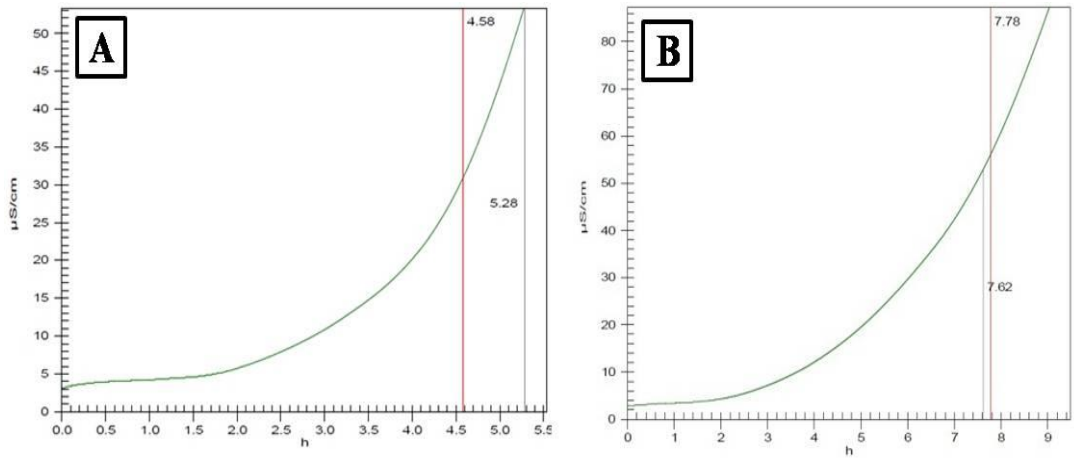


Fig. 7.19. Hypothetical reaction pathway of unsaturated fatty acid aromatization process

7.2.5. Comparison of the Effect of Natural and Marine Antioxidant Blend Added to Purified Fatty Acid Methyl Esters in an Accelerated Stability Life Study with Synthetic Antioxidants

7.2.5A. Oil Stability Index using Rancimat Analysis

In the case of rosemary and green tea blend (1 %) the oxidative stability of the purified fish methyl esters increased significantly ($p<0.05$) (IT = 4.58 h) (Table 7.10) as compared to the control (POA, 0.28 h) (Table 7.8). Wada and Fang (1992) reported a strong synergistic effect of the combination rosemary extract plus α-tocopherol of these antioxidants in sardine methyl esters. These authors suggested that rosemary extract acts as hydrogen atom donor, regenerating the α-tocopheroxyl radical to α-tocopherol. The seaweed blend constituted of KAF, HMF and JRF (0.1:0.2:0.2, w/w %) added to purified sardine fatty acid methyl esters at 1% showed rancimat induction time of 6.78h (Table 7.8) as compared to 4.58 h of natural herbal extract blend (POE). However the mix of seaweed blend, KAF + HMF + JRF (0.1:0.2:0.2, w/w %) and natural herbal extract blend (total at 1%) showed further elevation in IT with 7.78h (POF, Table 7.10, Fig. 7.20). Both the synthetic antioxidants, α-tocopherol and BHT (POG & POH) at 0.5% concentration showed significantly lower IT as compared to that of POE and POF ($p<0.05$) (Table 7.10). Hence, it is evident that the combination of marine and herbal extracts significantly increased the oxidative stability of the purified sardine methyl esters.



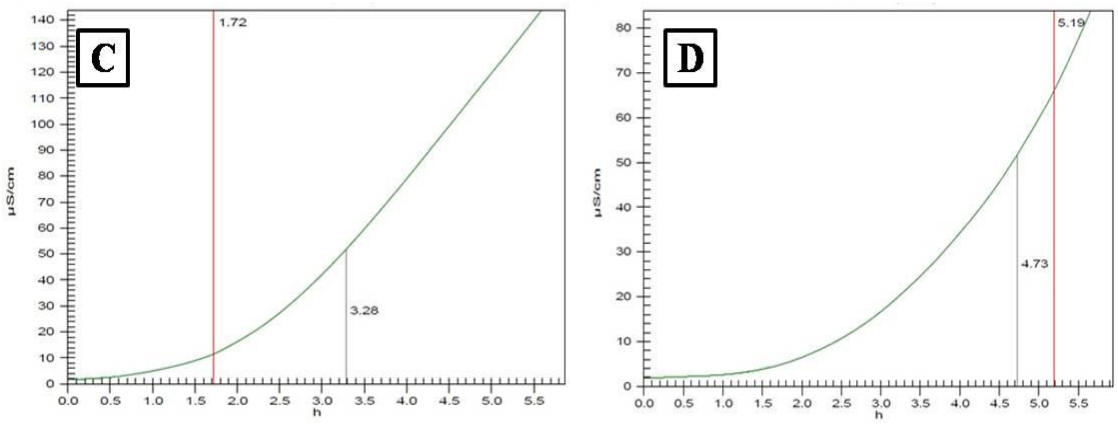


Fig. 7.20. Oil stability indices of the (**A**) PO added with natural antioxidant blend 1% POE; (**B**) PO added with marine antioxidant (0.5%) + natural extract (0.5%) POF; (**C**) PO added with α-tocopherol, POG and (**D**) PO added with BHT, POH

7.2.5B. P.V., pA.V., TOTOX, TBARS Values and DPPH Scavenging Activities during Accelerated Stability Study

POE showed significantly higher percent reduction of P.V. after 12 days (147.8 %) than that of POF (percent reduction of 89.9 %) (Table 7.10, Fig. 7.21). However, the percent reduction of POG and POH added with synthetic antioxidants showed higher P.V. values (326 & 312.8 % reduction, respectively) after 12 days.

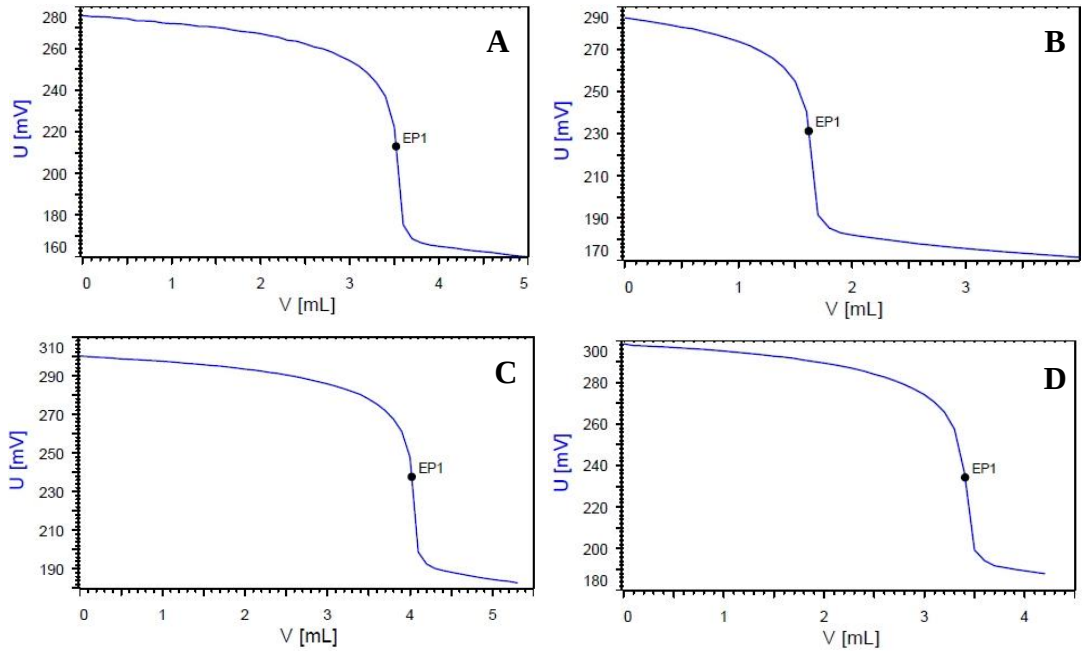


Fig. 7.21. Potentiometric titration curves obtained using an automatic titrator indicating the peroxide levels in; (**A**) POE at 12th day; (**B**) POF at 12th day; (**C**) POG at 12th day; (**D**) POH at 12th day

The pA.V. of POE increased to 38 at 12th day and to 29.3 for POF. On the other hand, POG and POG exhibited a pA.V. of 32 and 30 after 12 days (Table 7.10).

A significantly higher TOTOX values were observed for POG (181.6) followed by POG (163.2), POE (127.8) and POF (98.6) (Table 7.10).

TBARS values were found to be higher for POG after 12 days of shelf life study (0.78 MDAEQ/kg). However, the purified fatty acid methyl esters mixed with both natural and marine antioxidants (POF) showed minimum TBARS value after 12 days of accelerated shelf life study (0.53 MDAEQ/kg) (Table 7.10). POE showed comparable values with that POH, while POF showed lower TBARS value as compared with α-tocopherol and BHT after 12 days (Table 7.10).

The DPPH scavenging activity results showed that POE and POF might be potential antioxidants, and their activities were equivalent or higher than that of α-tocopherol and BHT (Table 7.10).

7.2.5C. Fatty acid composition during the accelerated shelf life study of purified fatty acid methyl esters added with herbal combination, marine antioxidant and herbal combinations

Table 7.11 shows the variations in the fatty acid profile of purified methyl esters added with the herbal blend, rosemary and green tea (POE), marine antioxidant blend plus herbal blend of rosemary and green tea (POF), α-tocopherol (POG) and BHT (POH). The percent reduction (w.r.t day 0) in EPA was found to be higher for POE (40.4% reduction) as compared with POF (34 % reduction), which showed lower reduction than α-tocopherol (POG 36.6%) (Fig. 22). It is interesting to note that BHT was not able to prevent DHA from oxidative degradation as compared to that of POF and POE (Fig. 7.23). POE added with the herbal blend showed considerable reduction in the DHA content as compared to POF (marine combination plus herbal blend). This demonstrated that the marine and herbal combination might synergistically worked out to prevent the oxidative rancidity of sardine fatty acid methyl esters. A schematic diagram illustrating lipid peroxidation inhibitory and antioxidant mechanism of rosemary active principles (Fig. 7.23).

Table 7.10 Quality parameters of purified methyl esters added with different treatments (POE, POF, POG and POH)

		POE	POF	POG	POH
IT		4.58	7.78	1.72	4.73
ST		5.28	7.62	3.28	5.19
AAI		16.36	27.79	6.14	16.89
P.V.	0	18.14 ± 0^{a}	18.25 ± 0^{a}	17.54 ± 0^{a}	16.14 ± 0^{a}
	4	29.32 ± 0^{b}	28.56 ± 0^{b}	24.29 ± 0^{b}	25.06 ± 0^{b}
	8	36.25 ± 0^{c}	31.11 ± 0^{bc}	47.24 ± 0^{c}	49.64 ± 0^{c}
	12	66.62 ± 0^{d}	34.66 ± 0^{c}	74.71 ± 0^{d}	68.12 ± 0^{d}
PA.V.	0	19.25 ± 0^{a}	18.01 ± 0^{a}	21.22 ± 0^{a}	18.25 ± 0^{a}
	4	25.45 ± 0^{b}	25.21 ± 0^{b}	23.25 ± 0^{a}	20.01 ± 0^{a}
	8	30.68 ± 0^{b}	27.65 ± 0^{b}	30.01 ± 0^{b}	22.14 ± 0^{a}
	12	37.99 ± 0^{c}	29.31 ± 0^{b}	32.14 ± 0^{b}	30.01 ± 0^{b}
TOTOX	0	55.53 ± 0^{a}	54.51 ± 0^{a}	56.3 ± 0^{a}	50.53 ± 0^{a}
	4	84.09 ± 0^{b}	82.33 ± 0^{b}	71.83 ± 0^{a}	70.13 ± 0^{b}
	8	103.18 ± 0^{c}	89.87 ± 0^{c}	124.49 ± 0^{a}	121.42 ± 0^{c}
	12	127.89 ± 0^{d}	98.63 ± 0^{d}	181.56 ± 0^{a}	163.25 ± 0^{d}
TBARS	0	0.48 ± 0^{a}	0.44 ± 0^{a}	0.65 ± 0^{a}	0.54 ± 0^{a}
	4	0.49 ± 0^{a}	0.5 ± 0^{a}	0.62 ± 0^{a}	0.59 ± 0^{a}
	8	0.56 ± 0^{a}	0.51 ± 0^{a}	0.71 ± 0^{a}	0.64 ± 0^{a}
	12	0.66 ± 0^{a}	0.53 ± 0^{a}	0.78 ± 0^{a}	0.67 ± 0^{a}
DPPH	0	99.01 ± 0^{a}	99.89 ± 0^{a}	96.25 ± 0^{a}	99.02 ± 0^{a}
	4	94.25 ± 0^{bc}	96.25 ± 0^{a}	91.02 ± 0^{a}	97.02 ± 0^{a}
	8	90.26 ± 0^{cd}	91.21 ± 0^{ab}	84.25 ± 0^{b}	86.32 ± 0^{b}
	12	86.21 ± 0^{d}	90.01 ± 0^{b}	78.25 ± 0^{b}	84.25 ± 0^{b}

POE – POA + Natural extract blend (1%); POF – POA + Natural extract blend (0.5%) + COT_3 (0.5%), POG - POA + 0.5% α-tocopherol; POH – POA + 0.5% BHT. IT – Induction time in h; ST – Stability time in h; P.V. – Peroxide value represented in $meqO_2/kg$; pA.V. – p-anisidine value; TOTOX – total oxidation value (2 x P.V. + pA.V.); TBARS – Thiobarbituric acid reactive species represented in mg MDA equivalent compounds/kg sample (MDAEQ/kg); DPPH – free radical scavenging activity(%).

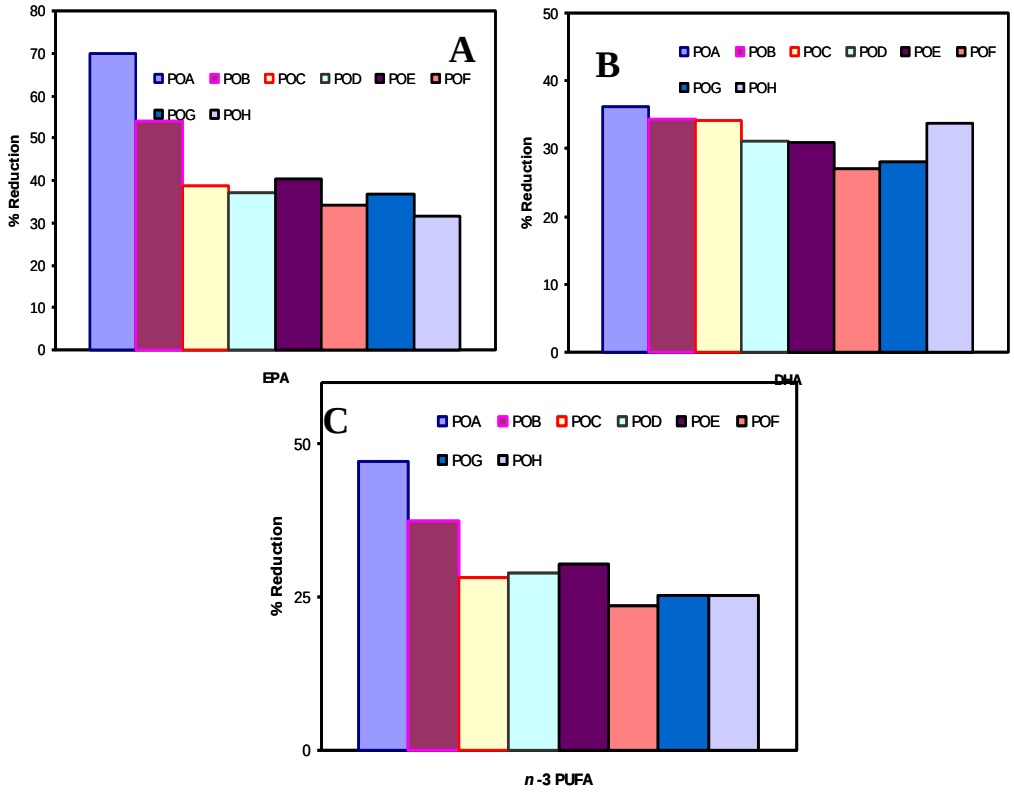


Fig. 7.22. Percent reduction in (**A**) EPA, (**B**) DHA and (**C**) ∑*n*-3PUFA after 12 days of accelerated shell life study of different treatments $COT_{1\text{-}9}$

Table 7.11 Fatty acid composition of POE-POH during accelerated stability life study of 12 days

Fatty acids	POE				POF				POG				POH			
	0	4	8	12	0	4	8	12	0	4	8	12	0	4	8	12
Saturated																
14:0	0.09±0.01	0.07±0.01	0.06±0.01	0.02±0	0.09±0.01	0.06±0.01	0.07±0.01	0.01±0	0.01±0	0.06±0.01	1.01±0.1	0.89±0.09	0.01±0	0.02±0	0.02±0	0.09±0.01
15:0	0.01±0	0.1±0.01	0.26±0.03	0.39±0.04	0.05±0.01	0.14±0.01	0.26±0.03	0.11±0.01	ND	0.1±0.01	0.26±0.03	0.39±0.04	ND	0.1±0.01	0.26±0.03	0.39±0.04
16:0	0.01±0	0.01±0	0.35±0.04	1.2±0.12	0.02±0	0.05±0.01	0.36±0.04	1.01±0.1	ND	0.01±0	0.36±0.04	0.87±0.09	ND	0.01±0	0.36±0.04	0.88±0.09
17:0	0.01±0	0.04±0	0.36±0.04	0.61±0.06	0.01±0	0.04±0	0.32±0.03	0.41±0.04	0.01±0	0.04±0	0.32±0.03	0.56±0.06	0.01±0	0.04±0	0.32±0.03	0.56±0.06
18:0	0.01±0	0.11±0.01	0.15±0.02	0.3±0.03	0.01±0	0.11±0.01	0.16±0.02	0.3±0.03	0.01±0	0.11±0.01	0.16±0.02	0.05±0.01	0.01±0	0.11±0.01	0.16±0.02	0.34±0.03
20:0	0.04±0	0.12±0.01	0.12±0.01	0.3±0.03	0.04±0	0.07±0.01	0.13±0.01	0.25±0.03	0.04±0	0.08±0.01	0.13±0.01	0.06±0.01	0.04±0	0.08±0.01	0.13±0.01	0.43±0.04
22:0	0.06±0.01	0.05±0.01	0.04±0	0.14±0.01	0.06±0.01	0.01±0	0.04±0	0.11±0.01	0.04±0	0.01±0	0.04±0	0.06±0.01	0.04±0	0.01±0	0.04±0	0.14±0.01
24:0	0.17±0.02	0.18±0.02	0.23±0.02	0.59±0.06	0.17±0.02	0.21±0.02	0.23±0.02	0.46±0.05	0.19±0.02	0.21±0.02	0.23±0.02	0.04±0	0.19±0.02	0.21±0.02	0.23±0.02	0.58±0.06
∑SFA	0.4±0.04	0.68±0.07	1.57±0.16	3.55±0.36	0.45±0.05	0.69±0.07	1.57±0.16	2.66±0.27*	0.3±0.03	0.62±0.06	2.51±0.25	2.92±0.29	0.3±0.03	0.58±0.06	1.52±0.15	3.41±0.34
Monounsaturated																
14:1*n*7	0.17±0.02	0.16±0.02	0.24±0.02	0.58±0.06	0.17±0.02	0.14±0.01	0.24±0.02	0.58±0.06	0.01±0	0.16±0.02	0.22±0.02	0.57±0.06	0.01±0	0.16±0.02	0.24±0.02	0.58±0.06
15:1*n*7	0.02±0	0.01±0	0.01±0	0.1±0.01	0.02±0	0.01±0	0.01±0	0.1±0.01	0.01±0	0.01±0	0.01±0	0.1±0.01	0.01±0	0.01±0	0.01±0	0.1±0.01
16:1*n*7 *trans*	0.01±0	0.02±0	0.02±0	0.06±0.01	0.01±0	0.04±0	0.02±0	0.03±0	ND	0.02±0	0.02±0	0.03±0	ND	0.01±0	0.02±0	0.03±0
16:1*n*7	0.33±0.03	1.2±0.12	8.34±0.83	11.36±1.14	0.33±0.03	1.2±0.12	7.02±0.7*	10.02±1*	0.43±0.04	1.2±0.12	6.56±0.66*	11.35±1.14*	0.44±0.04	1.2±0.12	8.34±0.83*	12.25±1.23*
18:1*n*9 *trans*	ND	0.01±0	0.08±0.01	0.12±0.01	ND	0.01±0	0.02±0	0.2±0.02	ND	0.01±0	0.08±0.01	0.12±0.01	ND	0.01±0	0.08±0.01	0.12±0.01
18:1*n*9	0.56±0.06	0.99±0.1	1.65±0.17	1.06±0.11	0.56±0.06	0.98±0.1	1.89±0.19	1.21±0.12	0.58±0.06	0.98±0.1	1.56±0.16	3.21±0.32	0.56±0.06	0.99±0.1	2.01±0.2	3.21±0.32
20:1*n*9	0.02±0	0.06±0.01	0.09±0.01	0.12±0.01	0.02±0	0.05±0.01	0.09±0.01	0.56±0.06	0.03±0	0.08±0.01	0.08±0.01	0.12±0.01	0.03±0	0.06±0.01	0.09±0.01	0.12±0.01
22:1*n*9	0.34±0.03	0.36±0.04	0.91±0.09	1.18±0.12	0.34±0.03	0.32±0.03	0.91±0.09	1.56±0.16	0.34±0.03	0.45±0.05	0.89±0.09	1.18±0.12	0.34±0.03	0.36±0.04	0.91±0.09	1.18±0.12
24:1*n*9	0.42±0.04	0.53±0.05	0.32±0.03	0.22±0.02	0.42±0.04	0.14±0.01	0.32±0.03	0.22±0.02	0.43±0.04	0.58±0.06	0.45±0.05	0.22±0.02	0.43±0.04	0.53±0.05	0.32±0.03	0.22±0.02
∑MUFA	1.87±0.19	3.34±0.33	11.66±1.17*	14.8±1.48*	1.87±0.19	2.89±0.29	10.52±1.05*	14.48±1.45*	1.83±0.18	3.49±0.35	9.87±0.99*	16.9±1.69*	1.82±0.18	3.33±0.33	12.02±1.2*	17.81±1.78*
Polyunsaturated																
18:2*n*6 *trans*	0.04±0	0.06±0.01	0.12±0.01	0.01±0	0.01±0	0.02±0	0.02±0	0.02±0	ND	0.01±0	0.2±0.02	0.03±0	ND	0.02±0	0.06±0.01	0.07±0.01
18:2*n*6 *cis*	3.15±0.32	4.98±0.5	4.56±0.46	4.27±0.43	5.35±0.54	4.06±0.41	3.86±0.39	3.09±0.31	5.28±0.53	4.06±0.41	3.86±0.39	3.09±0.31	5.28±0.53	4.06±0.41	3.87±0.39	3.09±0.31
18:3*n*6 *trans*	ND	ND	ND	ND	ND	ND	ND	0.02±0	0.09±0.01	0.78±0.08	0.04±0	0.19±0.02	ND	0.01±0	0.02±0	0.04±0
18:3*n*6 *cis*	0.48±0.05	0.78±0.08	0.64±0.06	0.24±0.02	0.48±0.05	0.78±0.08	0.64±0.06	0.46±0.05	0.65±0.07	0.78±0.08	0.64±0.06	0.46±0.05	0.65±0.07	0.78±0.08	0.65±0.07	0.47±0.05
18:3*n*3	0.05±0.01	0.06±0.01	0.09±0.01	0.12±0.01	0.05±0.01	0.06±0.01	0.09±0.01	0.12±0.01	0.03±0	0.06±0.01	0.09±0.01	0.12±0.01	0.03±0	0.06±0.01	0.09±0.01	0.12±0.01
20:2*n*6	3.12±0.31	3.13±0.31	3.26±0.33	3.35±0.34	3.12±0.31	2.01±0.2	1.86±0.19	1.47±0.15	3.06±0.31	2.21±0.22	1.98±0.2	1.48±0.15	3.06±0.31	2.01±0.2	1.85±0.19	1.52±0.15
20:3*n*6	0.15±0.02	0.01±0	0.23±0.02	0.17±0.02	0.15±0.02	0.01±0	0.23±0.02	0.17±0.02	0.1±0.01	0.01±0	0.23±0.02	0.17±0.02	0.1±0.01	0.01±0	0.23±0.02	0.17±0.02
20:4*n*6	0.38±0.04	0.38±0.04	0.38±0.04	0.39±0.04	0.38±0.04	0.38±0.04	0.38±0.04	0.39±0.04	0.37±0.04	0.38±0.04	0.38±0.04	0.39±0.04	0.37±0.04	0.38±0.04	0.38±0.04	0.39±0.04
20:5*n*3 *trans*	ND	0.04±0	0.06±0.01	0.12±0.01	ND	0.02±0	0.05±0.01	1.01±0.1	ND	0.04±0	0.06±0.01	1.04±0.1	ND	0.01±0	0.02±0	0.02±0
20:5*n*3 *cis*	47.05±4.71	42±4.2	38.69±3.87*	28.06±2.81*	47.02±4.7	42.05±4.21*	37.15±3.72*	31.02±3.1*	47.55±4.76	35.46±3.55*	26.21±2.62*	30.15±3.02*	47.56±4.76	42.36±4.24	38.05±3.81*	32.56±3.26*
22:5*n*3 *trans*	ND	0.05±0.01	0.06±0.01	0.1±0.01	ND	0.02±0	0.05±0.01	0.04±0	ND	0.01±0	0.02±0	0.03±0	ND	0.26±0.03	0.03±0	0.04±0
22:5*n*3 *cis*	2.01±0.2	4.86±0.49	5.02±0.5	6.21±0.62	2.01±0.2	4.86±0.49	5.02±0.5	7.77±0.78*	2.05±0.21	4.86±0.49	5.02±0.5	7.77±0.78	2.05±0.21	4.86±0.49	5.02±0.5	7.77±0.78
22:6*n*3 *trans*	0.01±0	0.41±0.04	0.96±0.1	1.06±0.11	0.01±0	0.06±0.01	0.12±0.01	0.16±0.02	ND	0.41±0.04	0.78±0.08	1.06±0.11	ND	0.02±0	0.02±0	0.05±0.01
22:6*n*3 *cis*	38.21±3.82	32.69±3.27	30.25±3.03*	26.41±2.64*	39±3.9	34.02±3.4	30.56±3.06*	28.44±2.84*	38.15±3.82	30.16±3.02*	28.71±2.87*	27.48±2.75*	38.15±3.82	30.16±3.02*	30.01±3*	25.26±2.53*
∑PUFA	94.65±9.47	89.45±8.95	84.32±8.43*	70.51±7.05*	97.58±9.76	88.35±8.84*	80.03±8*	74.18±7.42*	97.33±9.73	79.23±7.92*	68.22±6.82*	73.46±7.35*	97.25±9.73	85±8.5*	80.3±8.03*	71.57±7.16*
EPA+DHA	85.26±8.53	74.69±7.47*	68.94±6.89*	54.47±5.45*	86.02±8.6	76.07±7.61*	67.71±6.77*	59.46±5.95*	85.7±8.57	65.62±6.56*	54.92±5.49*	57.63±5.76*	85.71±8.57	72.52±7.25*	68.06±6.81*	57.82±5.78*
∑*n*3PUFA	87.32±8.73	79.61±7.96*	74.05±7.41*	60.8±6.08*	88.08±8.81	80.99±8.1*	72.82±7.28*	67.35±6.74*	87.78±8.78	70.54±7.05*	60.03±6*	65.52±6.55*	87.79±8.78	77.44±7.74*	73.17±7.32	65.71±6.57
∑*n*6PUFA	6.8±0.68	8.5±0.85	8.43±0.84	8.18±0.82	9±0.9	6.46±0.65	6.33±0.63	5.14±0.51	8.9±0.89	7.44±0.74	6.49±0.65	5.32±0.53	8.81±0.88	6.47±0.65	6.35±0.64	5.21±0.52
∑*n*3/∑*n*-6	12.84±1.28	9.37±0.94	8.78±0.88	7.43±0.74*	9.79±0.98	12.54±1.25	11.5±1.15	13.1±1.31	9.86±0.99	9.48±0.95	9.25±0.93	12.32±1.23	9.96±1	11.97±1.2	11.52±1.15	12.61±1.26
∑*n*6/∑*n*3	0.08±0.01	0.11±0.01	0.11±0.01	0.13±0.01	0.1±0.01	0.08±0.01	0.09±0.01	0.08±0.01	0.1±0.01	0.11±0.01	0.11±0.01	0.08±0.01	0.1±0.01	0.08±0.01	0.09±0.01	0.08±0.01
∑PUFA/∑SFA	236.63±23.66	131.54±13.15*	53.71±5.37*	19.86±1.99*	216.84±21.68	128.04±12.8*	50.97±5.1*	27.89±2.79*	324.43±32.44	127.79±12.78*	27.18±2.72*	25.16±2.52*	324.17±32.42	146.55±14.66*	52.83±5.28*	20.99±2.1*
∑TRANS	0.06±0.01	0.59±0.06	1.3±0.13	1.47±0.15	0.03±0	0.17±0.02	0.28±0.03	1.48±0.15	0.09±0.01	1.28±0.13	1.2±0.12	2.5±0.25	ND	0.34±0.03	0.25±0.03	0.37±0.04

Data are expressed as mean ± standard deviation of three replicates. ∑SFA Total saturated fatty acids, ∑MUFA Total monounsaturated fatty acids, ∑PUFA Total polyunsaturated fatty acids. * represents a statistical difference ($p<0.05$) between 0^{th} and 12^{th} day. ND: not detected. POA = PO - Purified oil obtained by Ag-silica chromatography (Chapter 5)

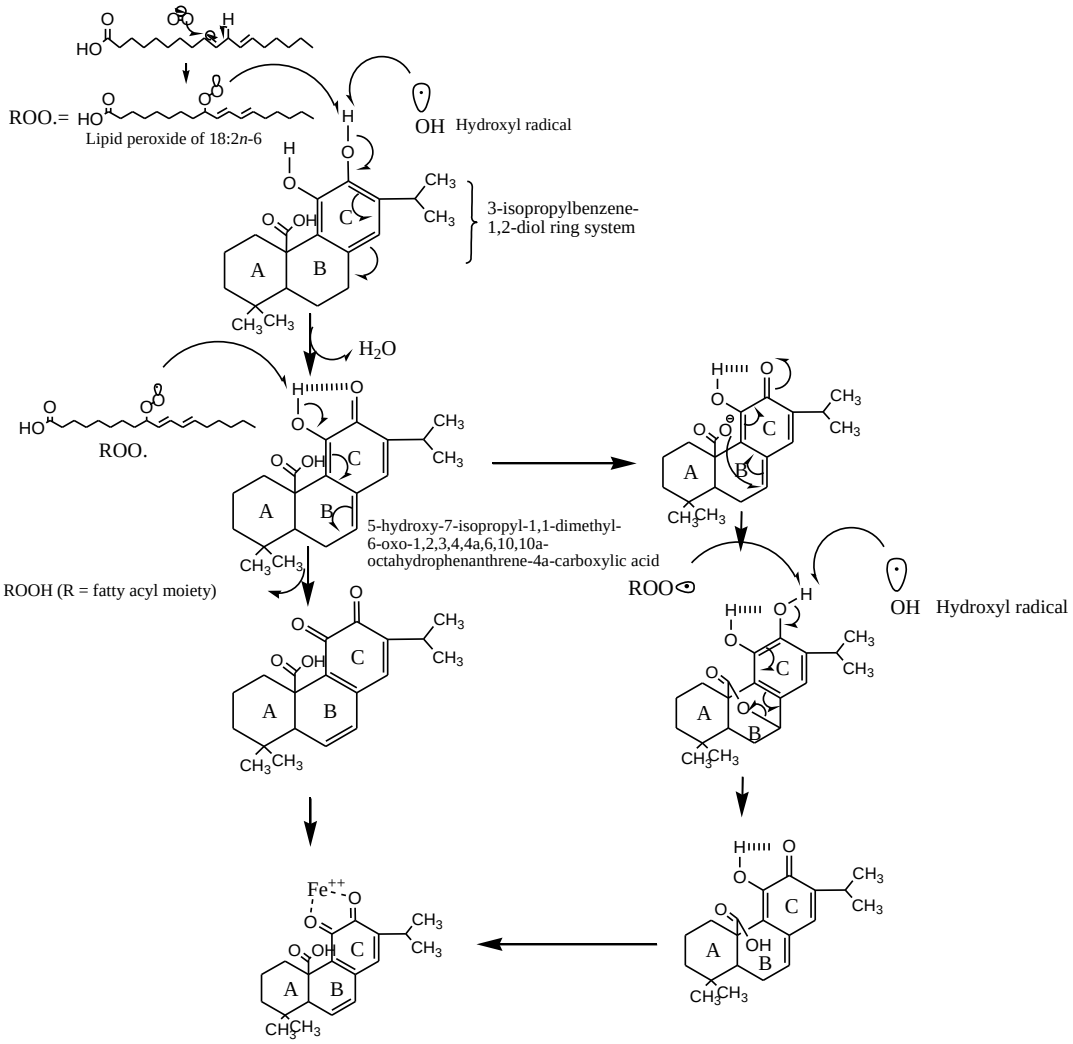


Fig.7.23. A schematic diagram illustrating lipid peroxidation inhibitory and antioxidant mechanism of rosemary active principles carnosic acid with 1-(1, 1-dimethylperhydro-4-naphthalenyl) -1-ethanone) and carnosol with perhydro-1-isochromenone and 3-isopropyl-1, 2-benzenediol, which together are responsible for ~90% of antioxidant activity. HAT (H-donation) and chain breaking mechanism, as in phenolic antioxidants, is the mode of action of carnosic acid and carnosol. Another mechanism by that these polyphenolic compounds exhibit lipid peroxidation is of metal (Fe^{2+}) ion chelation by phenoxyl radical and free hydrogen on -OH group. The polyphenols with *o*-dihydroxy groups provide improved stability to polyphenol radical by electron delocalization through the system accompanying radical formation.

The effective inhibition of lipid peroxidation in the treatments with antioxidative additives may be attributed to the presence of phenolic antioxidants in the additives that were reported to disrupt free-radical chain reaction by donating a proton to fatty acid radicals to terminate chain reactions, and have roles to inhibit lipid peroxidation. Though carnosic acid is the primary antioxidant in rosemary, and

it metabolized to other compounds like carnosol, rosamanol and rosamariqionone, which too were reported to possess antioxidant and lipid peroxidation inhibitory properties. Rosmarinic acid possesses 1, 2-dihydroxybenzene (catechol) ring system, contributing to its antioxidant activity. Furthermore, the presence of four phenolic hydrogens in 4-[(*E*) -1-propenyl] -1, 2-benzenediol moiety in rosmarinic acid significantly increased the polarity of the catechol ring system. These phenolic hydrogens undergo intramolecular H-bonding thereby improving its radical stability, and ease of H-atom abstraction from catechol forming phenoxyl radical. Another mechanism by that these polyphenolic compounds exhibit lipid peroxidation is of metal (Fe^{2+}) ion chelation by phenoxyl radical and free hydrogen on -OH group. The polyphenols with ortho-dihydroxy groups provide improved stability to polyphenol radical by electron delocalization through the system accompanying radical formation.

A possible explanation for this synergistic effect would be that a heterogeneous activation of a hydroxyl group on the antioxidant principles of other antioxidants to enhance the formation of H radical, which rapidly reacts with exogenous free radical (DPPH.) to quench it. A phenolic antioxidant bearing ortho-dihydroxyl groups as in carnosic acid (with 1-(1, 1-dimethylperhydro-4-naphthalenyl)-1-ethanone) and carnosol (with perhydro-1-isochromenone and 3-isopropyl-1, 2-benzenediol) in rosemary was investigated as efficient antioxidants, and the produced stable *o*-quinone material plays a key role in its antioxidant activity (Wei & Ho 2006).

7.3. Conclusions

The study also demonstrated the efficacy of the seaweed extracts, mainly, *K. alvarezii*, *H. musciformis* and *J. rubens* for use as antioxidative supplements to the refined oil (EPA 14.7 %; DHA 5.7 %; ∑PUFA 25.8 %), concentrated (EPA 33.4 %; DHA 27.8 %; ∑PUFA 73.9 %) and purified fatty acid methyl esters (EPA 47.6 %; DHA 38.2 %; ∑PUFA 97.3 %) in a time dependent accelerated shelf life study (for 12 days) with respect to their oil stability indices (induction time & stability time), peroxide, para-anisidine, TOTOX and TBARS values, free radical scavenging

activity along with the fatty acid composition. Both refined oil and fatty acid concentrate of sardines were demonstrated to be significantly protected by the *K. alvarezii*, *H. musciformis* and *J. rubens* extracts. The effect of the seaweed extract blend (especially, COT_3 prepared by mixing 0.1:0.2:0.2 % w/w of *K. alvarezii*, *H. musciformis* and *J. rubens* extracts, respectively) at 0.5 % level to the concentrated fatty acid methyl esters, showed a positive synergistic effect as calculated from its degree of synergism (% SYN 221.8 %). Furthermore, a threshold level of this blend COT_3 (1 % to purified fatty acid methyl esters) was found to be sufficient for optimum antioxidant activity, and further addition of this antioxidant blend (2 % to purified fatty acid methyl esters) does have no additional improvement on the stability of the essential long chain fatty acids. A significant reduction of *n*-3 PUFAs, and deficiency to arrest the isomeric conversion of *cis* PUFAs to their *trans* isomers of purified fatty acid methyl esters were realized, and this drawback was solved by the addition of the marine antioxidant combination. This combination further realized a comparable antioxidative effect as that of the herbal blend prepared from rosemary and green tea extracts. The quality of the oil/esters was also further validated by ^1H NMR spectroscopy conjugated with FT-IR functional group analyses, which led us to infer that the antioxidative natural additives play a major role in retaining the native form of polyunsaturated fatty acids for a longer period on shelf. These additives also proved to prevent the formation of undesirable small molecular weight aldehydes and aromatic compounds from the fatty acid precursors on shelf. This study demonstrated the optimized protocol to maintain the sardine oil/esters stabilized with marine additive to retain their functional properties for a longer period on shelf.

Chapter 8

ISOLATION AND PURIFICATION OF BIOACTIVE SECONDARY METABOLITES FROM POTENTIAL ADDITIVES

Contents

Background

Chemical compounds with oxidation-inhibiting properties are present in tropical sessile marine macro algae or seaweeds as a protective mechanism against oxidative stress factors in hyperhaline (more than 35 ppt) and oceanic ecosystems (Kim *et al.,* 2008; Chakraborty *et al.,* 2010). Marine macro algae evolved a multitude of defense mechanisms to deter oxidative stress related diseases in marine ecosystem by producing bioactive metabolites with antioxidative activities. These species typically lack the protection of an external shell with defensive mechanisms as means to shield themselves from oxidative stress conditions in the hostile oceanic ecosystem, and, therefore, have been the favorite choice of natural product chemists to isolate potential antioxidative lead molecules.

These salt-resistant species are the richest natural sources of high value bioactive compounds, many of which belong to novel chemical classes not found in terrestrial sources. This might explain the utilization potential of marine macro algae as renewable sources to offer excellent package of ‘natural’ antioxidative molecules. Marine macro algae also don’t require freshwater for their growth, and these species exclusively depend on saline water for their development and reproduction. Considering the underutilization of these organisms, development of any biologically useful products from marine macro algae has duel benefits-as health products and their commercial farming in coastal habitats, resulting in C- sequestration and C-

budgeting in a scenario where climate change may pose a serious threat in future. Fewer studies have considered biochemistry and antioxidative importance of these unexploited and underutilized species from the coastal habitats of India. It is, therefore, imperative to characterize these underutilized and unconventional resources with respect to their antioxidative properties intended for use as additives, so as to achieve the goals of effective utilization of underutilized marine resources, based on their abundance in nature and their sustainable utilization.

This work therefore envisages to screen the red seaweeds (Rhodophyta) belonging to *Kappaphycus alvarezii*, *Hypnea muscifomis* and *Jania rubens* from the coastline of India for lead molecules with antiradical activities. The knowledge on the structural features responsible for antioxidative bioactivities will guide us to synthesize the molecules in commercial scale for use as new generation antioxidant leads. The macro algae is available in abundance and the occurrence is throughout the year from the coastline of India, particularly the Gulf of Mannar region of South-east coast of India. Among the different marine additives studied (*Chapter 7*), *Kappaphycus alvarezii*, *Hypnea muscifomis* and *Jania rubens* were found to possess properties to enhance the oxidative stability of the fish oil. This seaweed extracts imparted protection against lipid degradation in the refined, concentrated as well as purified sardine oil. Hence, the purification of these crude extracts was carried out by repeated purifications with the aid of various chromatographic techniques. The structural characterization was done with the aid of various spectroscopic techniques. The pure compounds were further assayed to document its free radical scavenging activity, lipid peroxidation inhibitory activity and Fe^{2+} ion chelating potential.

8.1. Materials and Methods

8.1.1. Chemicals and Reagents

All chemicals were of analytical, spectroscopic or chromatographic reagent grade, and were obtained from E-Merck (Darmstadt, Germany). All reagents and chemical solvents used for products isolation were of analytical grade or higher.

8.1.2. Isolation and Purification of Secondary Metabolites

8.1.2.1. Isolation of Secondary Metabolites from *Kappaphycus alvarezii*

The dried *Kappaphycus alvarezii* powder (2 kg) was extracted three times with MeOH (50–60 °C, 3 h), filtered through Whatman No. 1 filter paper and the pooled filtrate was concentrated (50 °C) *in vacuo* to one-third volume, and then partitioned successively with *n*-hexane (150 ml ×3), DCM (150 ml ×3) and EtOAc (150 ml ×3), concentrated *in vacuo* to furnish *n*-hexane (1670 mg), DCM (5510 mg), and EtOAc (1670 mg) fractions, respectively. The EtOAc fraction (1200 mg) of the methanolic extract of *K. alvarezii* was subjected to vacuum liquid chromatography and eluted using a stepwise gradient system from *n*-hexane (100%) to EtOAc (100%) to obtain 9 column fractions (Schematic diagram 1). These column fractions were evaluated for antioxidant activities, with respect to DPPH scavenging activity (0.1 mg/ml), TBARS formation inhibitory activity (0.1 μg/ml) and Fe^{2+} ion chelating activity (0.1 mg/ml) and the fractions which showed significantly higher antioxidant activities were further purified by column chromatography or preparative TLC (P-TLC) using EtOAc:*n*-hexane or MeOH:$CHCl_3$ or MeOH:EtOAc as mobile phase when required. The schematic diagram showing the purification of *Kappaphycus alvarezii* extract is shown in Fig. 8.1.

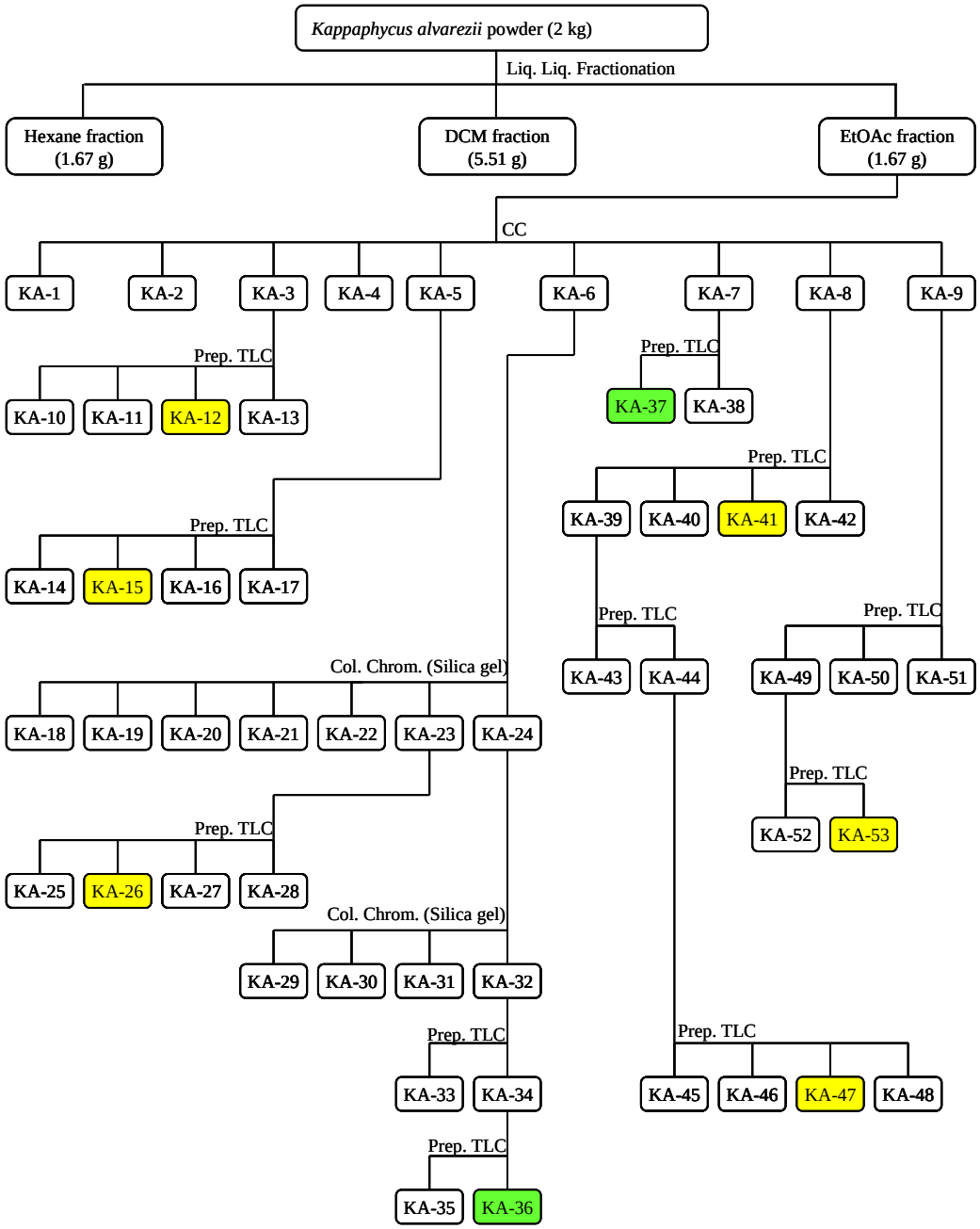


Fig. 8.1. Schematic diagram representing the chromatographic purification of the EtOAc fraction of *Kappaphycus alvarezii* (yellow highlight implies pure compound with higher activity but lower yield < 20mg; green highlight implies pure compound with higher activity & higher yield which considered for structural characterization)

8.1.2.2. Isolation of Secondary Metabolites from *Hypnea musciformis*

The dried *Hypnea musciformis* powder (1 kg) was extracted three times with MeOH (50–60 °C, 3 h), filtered through Whatman No. 1 filter paper and the pooled filtrate was concentrated (50 °C) *in vacuo* to one-third volume, and then partitioned

successively with *n*-hexane (150 ml ×3), DCM (150 ml ×3) and EtOAc (150 ml ×3), concentrated *in vacuo* to furnish *n*-hexane (13.05 g), DCM (9.93 g), and EtOAc (10.75 g) fractions, respectively. The EtOAc fraction (8000 mg) of the methanolic extract of *H. musciformis* was subjected to vacuum liquid chromatography and eluted using a stepwise gradient system comprising *n*-hexane/EtOAc/MeOH to obtain 8 column fractions (Schematic diagram Fig. 8.2). These column fractions were evaluated for antioxidant activities, with respect to DPPH scavenging activity (0.1 mg/ml), TBARS formation inhibitory activity (0.1 µg/ml) and Fe^{2+} ion chelating activity (0.1 mg/ml) and the fractions which showed significantly higher antioxidant activities were further purified by column chromatography or preparative TLC (P-TLC) or preparative HPLC (P-HPLC) using EtOAc:*n*-hexane or MeOH:$CHCl_3$ as mobile phase when required.

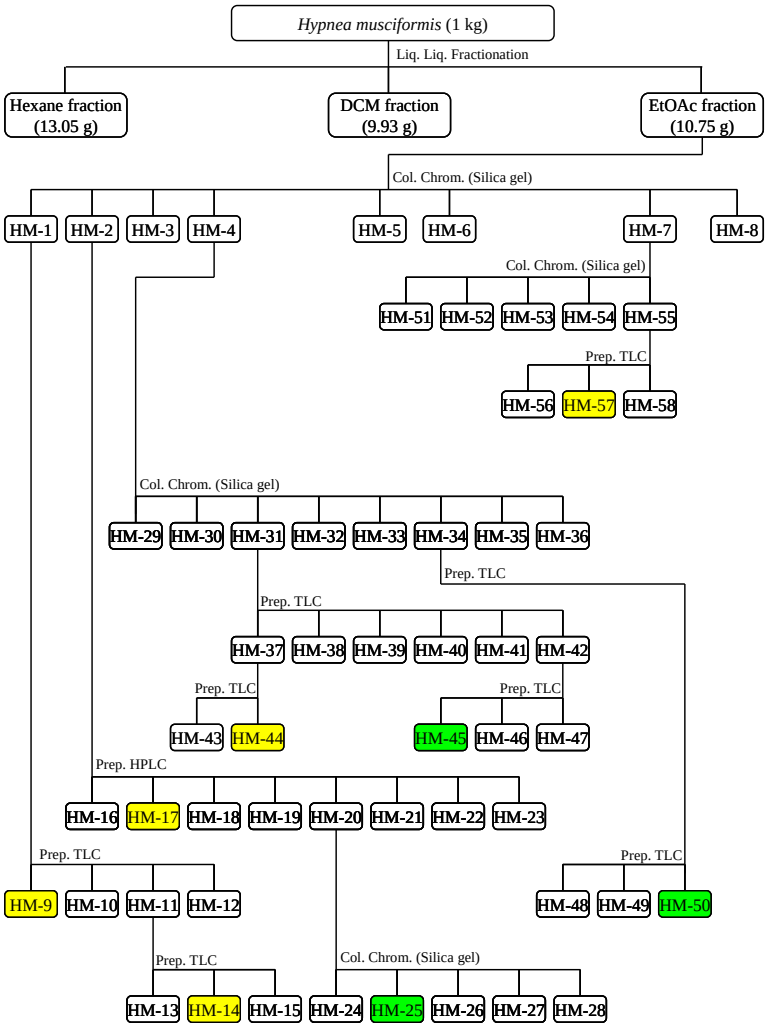


Fig. 8.2. Schematic diagram representing the chromatographic purification of the EtOAc fraction of *Hypnea musciformis* (yellow highlight implies pure compound with higher activity but lower yield < 20mg; green highlight implies pure compound with higher activity & higher yield which considered for structural characterization)

8.1.2.3. Isolation of Secondary Metabolites from *Jania rubens*

The dried *Jania rubens* powder (1 kg) was extracted three times with MeOH (50–60 °C, 3 h), filtered through Whatman No. 1 filter paper and the pooled filtrate was concentrated (50 °C) *in vacuo* to one-third volume, and then partitioned successively with *n*-hexane (150 ml ×3), DCM (150 ml ×3) and EtOAc (150 ml ×3), concentrated *in vacuo* to furnish *n*-hexane (13.94 g), DCM (10.57 g), and EtOAc (11.7 g) fractions, respectively. The EtOAc fraction of the methanolic extract of *J. rubens* (8000 mg) was subjected to vacuum liquid chromatography and eluted using a stepwise gradient system from *n*-hexane (100%) to EtOAc (100%) to obtain 9 column fractions (Schematic diagram Fig. 8.3). These column fractions were evaluated for antioxidant activities, with respect to DPPH scavenging activity (0.1 mg/ml), TBARS formation inhibitory activity (0.1 µg/ml) and Fe^{2+} ion chelating activity (0.1 mg/ml) and the fractions which showed significantly higher antioxidant activities were further purified by column chromatography or preparative TLC (P-TLC) using EtOAc:*n*-hexane or MeOH:$CHCl_3$ as mobile phase when required.

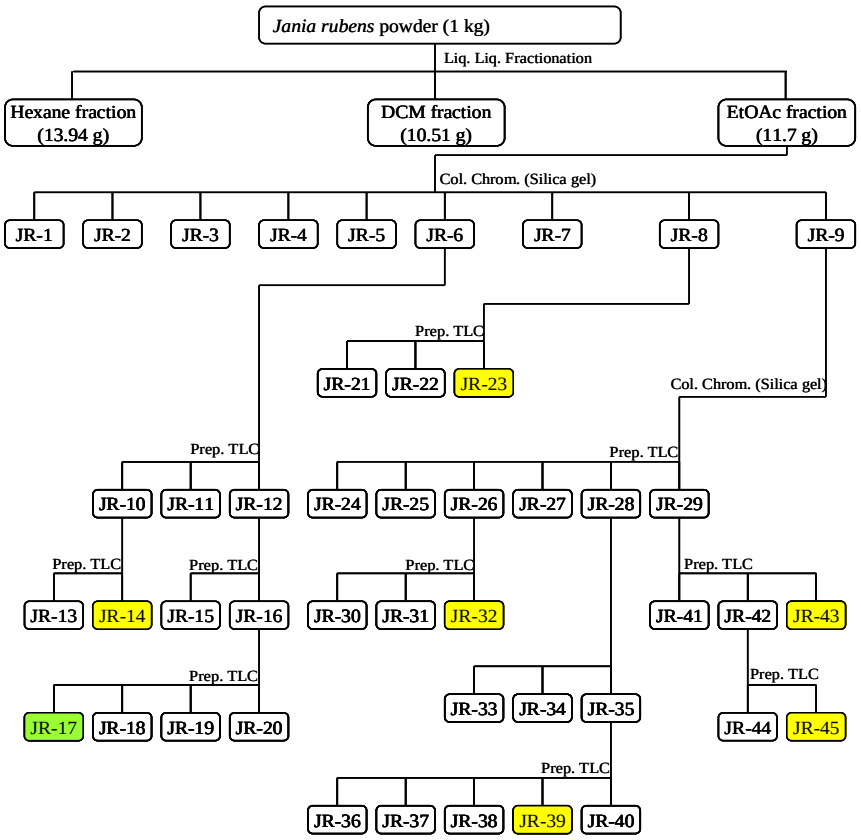


Fig. 8.3. Schematic diagram representing the chromatographic purification of the EtOAc fraction of *Jania rubens* (yellow highlight implies pure compound with higher activity but lower yield < 20mg; green highlight implies pure compound with higher activity & higher yield which considered for structural characterization)

8.1.3. Analysis

8.1.3.1. Chromatographic Analyses

8.1.3.1A. Preparative Thin Layer Chromatography (TLC)

TLC was performed on pre-coated TLC plates with silica gel 60 F_{254} (layer thickness 0.2 mm; 20 x 20 cm; Merck KGaA, Darmstadt, Germany) using different solvent systems as needed. The band separation on TLC was detected under UV lamp at 254 and 366 nm.

8.1.3.1B. Vacuum Column Chromatography (CC)

Normally, the columns were dry silica gel GF_{254} pre-packed, of 18 cm height and inner diameter of 12 cm, vertically clamped. The column is filled and saturated with the desired non-polar solvent in the mobile phase (eg: hexane) just prior to sample loading. Samples are dissolved in a small volume of the same solvent used and the resulting mixture was then packed onto the top of the column using special syringe. Using step gradient elution with non-polar solvent (here, *n*-hexane or DCM) and increasing amounts of polar solvents (e.g. EtOAc or MeOH) successive fractions were collected. The mobile phase (gradient elution) was pumped through the column with the help of air pressure resulting in sample separation.

8.1.3.2. Spectroscopic Analyses

8.1.3.2A.Fourier Transform Infra Red spectrometer (FTIR)

Fourier Transform Infra Red spectrometer (FTIR) spectra of the compounds under KBr pellets were recorded in a Thermo Nicolet, Avatar 370. The scanning was conducted in to mid IR range, i.e., between 4000-400cm^{-1}. UV spectra were obtained on a Varian Cary 50 UV-VIS spectrophotometer (Varian Cary, USA).

8.1.3.2B. Mass Spectrometry (MS) and Elemental Analysis

The Gas Chromatography-Mass Spectrometry (GC-MS) analyses were performed in electronic impact (EI) ionization mode in a Varian GC (CP-3800) interfaced with a Varian 1200L single quadruple Mass Spectrometer. ESI-MS spectra were acquired in the positive and negative modes with a turboionspray voltage,

curtain gas, turbo temperature, and nebulizer gas of -4500 V, 30 psi, 500 °C, and 50 psi (positive mode) at a flow rate of 1.5 ml/min. Elemental analysis of the compounds was carried out using a Euro Vector elemental analyzer (model no. EA3011). Liquid chromatography–mass spectrometry experiments were performed on an Applied Biosystems QTrap 2000 (Applied Biosystems, Darmstadt, Germany) coupled to an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) using a Luna 5 μ C_{18} column (100 A, 100 × 4.6 mm, Phenomenex, Aschaffenburg, Germany) or a Luna 3 μ C_{18} column (100 A, 50 × 1.0 mm, Phenomenex, Aschaffenburg, Germany) and a gradient of solvents A (0.1% HCOOH) and B (CH_3CN + 0.1% HCOOH; gradient 0% B to 100% B in 10 min) with a flow rate of 1.5 ml/min or 60 µl/min, respectively. ESI-MS spectra were acquired in the positive and negative modes with a turboion spray voltage, curtain gas, turbo temperature, and nebulizer gas of -4500 V, 30 psi, 500 °C, and 50 psi (positive mode, flow rate at 1.5 ml/min. The exact molecular ion weights of the pure compounds have been acquired by direct injection in a high resolution mass spectrometer, and were compared with the MarinLit database (Royal Society of Chemistry, London, Burlington House, London W1J 0BA) dedicated to marine natural products.

8.1.3.2C. Nuclear Magnetic Resonance Spectroscopy (NMR)

The ^1H and ^{13}CNMR spectra were recorded on a Bruker AVANCE III 500 MHz (AV 500) DRX 500 NMR spectrometer (Bruker, Karlsruhe, Germany) in $CDCl_3$ as aprotic solvent at ambient temperature with TMS as the internal standard (δ 0 ppm) equipped with 5 mm probes. The number of attached protons for the ^{13}CNMR signals was determined from DEPT experiments. Standard pulse sequences were used for Distortionless Enhancement by Polarization Transfer (DEPT), ^1H–^1H Correlation spectroscopy (^1H–^1H COSY for determining basic connectivity via J couplings through-bond), Nuclear Overhauser Effect Spectroscopy (NOESY for see through-space and conformation and for determining proximity of adjacent spin systems), Heteronuclear Single Quantum Correlation (HSQC for determining the narrower resonances for ^1H - ^{13}C correlations), and Heteronuclear Multiple Bond Correlation (HMBC to correlate X-nucleus shifts that are typically 2-4 bonds away from a proton) experiments.

8.1.3.3. Determination of Antioxidant Activities of the Purified Fractions

The DPPH scavenging activity (0.1 mg/ml), TBARS formation inhibitory activity (0.1 μg/ml) and Fe^{2+} ion chelating activity (0.1 mg/ml) of the column/TLC purified fractions were performed using the protocols explained in *Chapter 6*.

8.2. Results and Discussion

8.2.1. Secondary Metabolites from *Kappaphycus alvarezii*

The yield, antioxidant activities of each column/P-TLC fractions are given in Table 8.1. The R_f of all the P-TLC fractions are also shown in Table 8.1. Among the column fractions obtained from the EtOAc fraction of the MeOH extract, the fractions KA-3 (105.6 mg), KA-5 (174.3 mg), KA-6 (170.3 mg), KA-7 (196.1 mg), KA-8 (212.1 mg) and KA-9 (200.1 mg) exhibited significantly higher antioxidant activity with respect to scavenge DPPH radicals (64.3, 70, 61.4, 42.4, 64.3 & 60.3 %, respectively) ($p<0.05$). Among these fractions, KA-7 showed significantly higher Fe^{2+} ion chelating activity (28.5 %) followed by KA-3 and KA-9 (~ 10.2 %) ($p<0.05$). KA-6 exhibited maximum lipid peroxidation inhibitory activity (0.11 MDAEQ/kg) followed by KA-8, KA-7 and KA-5 (< 1.1 MDAEQ/kg).

KA-3 was further purified using p-TLC (10% EtOAc:*n*-hexane) to get a pure active compound, KA-12 (Rf 0.52) with low yield (2.1 mg). Similarly, KA-5 was further purified using p-TLC (10%EtOAc:*n*-hexane) to get the pure active compound, KA-15 (Rf 0.26), however with low yield (19.24 mg).

The fraction, KA-6 was purified using CC (*n*-hexane/EtOAc) to obtain 7 fractions, KA-18 through KA-24. The sub-fraction, K-23 which found to have 80.1 % DPPH radical scavenging activity and 9.3 % Fe^{2+} ion chelating activity was further purified using p-TLC (20% EtOAc:*n*-hexane) to obtain an active pure compound (KA-26) with 60.3 % DPPH radical scavenging activity. However the yield was found to be low for KA-26 (7.7 mg, Rf 0.34). KA-24 when purified using CC ($CHCl_3$:MeOH) yielded four fractions, KA-29 to KA-32 in which KA-32 (38.6 mg) registered 70.2 % DPPH radical scavenging activity and 4.2 % Fe^{2+} ion

chelating activity. KA-32 on subsequent purifications with PTLC was found to yielded an active pure compound, KA-36 (COMPOUND I, 25 mg).

The fraction, KA-7 which exhibited high radical scavenging ability as obtained from its DPPH radical scavenging ability (42.4 %), lipid peroxidation inhibitory activity (0.68 MDAEQ/kg) and Fe^{2+} ion chelating ability (28.5 %) was further chromatographed using p-TLC (20%EtOAc:*n*-hexane) to obtain the pure compound, KA-37 (COMPOUND II, 61.6 mg).

KA-8 on subsequent purifications using p-TLC in 10% MeOH:$CHCl_3$, 10% MeOH:$CHCl_3$ and 30% EtOAc:MeOH derived KA-41 and KA-47 with 62.3 and 74.5 % DPPH radical scavenging activity and 0.23 MDAEQ/kg lipid peroxidation inhibitory activity. However, the yield obtained for these compounds are negligible (~13 mg).

KA-9 on chromatographic purification using p-TLC (5% MeOH: EtOAc) yielded three fractions, KA-49, KA-50 and KA-51. KA-49 was further purified using p-TLC (10% MeOH: EtOAc) to obtain KA-53 (4.25 mg, R_f 0.9) with 74.3 % DPPH radical scavenging activity and 0.46 MDAEQ/kg lipid peroxidation inhibitory activity.

Table 8.1 Yield, R_f, DPPH radical scavenging activity, lipid peroxidation inhibitory activity (TBARS formation inhibitory activity) and Fe^{2+} ion chelating activity (%) of the fractions obtained by the purification of *Kappaphycus alvarezii*

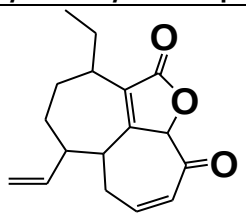
	Yield (mg)	R_f	DPPH radical scavenging activity	TBARS formation inhibitory activity	Fe^{2+} ion chelating activity
KA EtOAc fraction (CC E/H)					
KA-1 (100% H)	9.44		7.4		
KA-2 (1% E/H)	56.44		2.01	3.04	10.46
KA-3 (5% E/H)	105.64		64.31	0.48	10.24
KA-4 (10% E/H)	71.25		0.01	7.24	1.24
KA-5 (15% E/H)	174.25		70	1.07	1.06
KA-6 (20% E/H)	170.3		61.4	0.11	24.6
KA-7 (30% E/H)	196.11		42.36	0.68	28.46
KA-8 (50% E/H)	212.11		64.35	0.67	0.66
KA-9 (100% E)	200.1		60.26	2.36	10.16
KA-3 (PTLC 10% E/H)					
KA-10	18.25	0.14	14.1	NA	NA
KA-11	66.14	0.36	40.1	0.81	23.14
KA-12	2.1	0.52	84.4	NA	NA
KA-13	16.66	0.91	16.2	NA	NA
KA-5 (PTLC 10% E/H)					
KA-14	31.02	0.17	17.31	5.27	9.94
KA-15	19.24	0.26	68.24	0.91	10.01

KA-16	84.01	0.54	31.11	4.86	3.11
KA-17	36.25	0.96	0.26	8.44	0.11
KA-6 (CC E/H)					
KA-18 (100% H)	4.11	0.08	10.26	NA	NA
KA-19 (2% E/H)	2.56	0.17	44.11	NA	NA
KA-20 (5% E/H)	0.36	0.28	10.66	NA	NA
KA-21 (10% E/H)	1.25	0.53	50.11	NA	NA
KA-22 (20% E/H)	10.01	0.71	56.26	3.46	10.24
KA-23 (50% E/H)	80.01	0.8	80.12	1.34	9.26
KA-24 (100% E)	70.41	0.96	78.24	2.01	0.36
KA-23 (PTLC 20% E/H)					
KA-25	18.34	0.12	5.11	NA	NA
KA-26	7.66	0.34	60.31	0.26	31.11
KA-27	20.01	0.61	57.11	0.86	0.11
KA-28	31.33	0.68	0.01	6.11	0.44
KA-24 (CC MeOH/$CHCl_3$)					
KA-29 100% C	9.25	0.44	61.26	4.31	45.5
KA-30 2% M/C	10	0.61	50.1	0.31	0.02
KA-31 5% M/C	11.02	0.82	0.1	10.01	3.44
KA-32 10% M/C	38.55	0.9	70.16	2.11	4.16
KA-32 (PTLC 1% M/C)					
KA-33	10.02	0.46	50.01	2.11	0.11
KA-34	26.56	0.84	65.11	0.11	0.31
KA-34 (PTLC 1% M/C)					
KA-35	0.88	0.61	10.26	NA	NA
KA-36 COMPOUND I	25	0.81	82.11	0.99	3.11
KA-7 (PTLC 20% E/H)					
KA-37 COMPOUND II	61.56	0.54	69.11	0.11	41.01
KA-38	125.25	0.86	11.01	5.26	4.41
KA-8 (PTLC 10% M/C)					
KA-39	115.69	0.22	70.11	0.11	3.16
KA-40	31.21	0.46	31.04	0.16	10.21
KA-41	12.99	0.16	62.24	0.23	40.16
KA-42	50	0.95	40.06	2.11	0.04
KA-39 (PTLC 10% M/C)					
KA-43	15.11	0.46	40.24	1.06	1.11
KA-44	92.14	0.88	69.11	0.97	40.46
KA-44 (PTLC 30% E/H)					
KA-45	18.69	0.26	30.01	0.96	54.11
KA-46	31.02	0.45	0.06	4.24	1.06
KA-47	12.36	0.68	74.46	0.23	4.06
KA-48	24.55	0.9	18.46	2.13	0.16
KA-9 (PTLC 5% M/E)					
KA-49	100	0.12	80.06	4.31	10.16
KA-50	19.67	0.46	0.11	0.36	11.26
KA-51	79.21	0.61	4.46	5.24	1.06
KA-49 (PTLC 10% M/E)					
KA-52	73.44	0.6	18.16	6.46	0.16
KA-53	4.25	0.9	74.31	NA	NA

DPPH radical scavenging activity at 0.1 mg/ml is expressed in % (48 h); lipid peroxidation inhibitory activity at 0.1 μg/ml is expressed in MDAEQ/kg sample; Fe^{2+} ion chelating activity at 0.1 mg/ml is expressed in %. NA – NOT ASSAYED ie. the fractions with low yield were evaluated only for DPPH radical scavenging activity. CC – Column chromatography; PTLC -Preparative thin layer chromatography; M - Methanol; E - Ethyl acetate; H - *n*-hexane; C - Chloroform; KA - *Kappaphycus alvarezii*

8.2.1.1. Structural Characterization of Compound I (KA-36)

COMPOUND I (KA-36)

(8Z)-3-Ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione	
	
Yield	25 mg (2.08 %*)
Physical description	Greenish oil
Molecular formula	$C_{17}H_{20}O_3$
Molecular weight	272.4568

* Yield with respect to EtOAc fraction

The pure active COMPOUND I (KA-36) was found to be as (8Z)-3-ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione.

Another antioxidative compound isolated from this species was methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate. Interestingly, the structure of (8Z)-3-ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione (COMPOUND I) is similar to methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate, excepting the presence of 4,5-dihydro-8aH-cyclohepta[b]furan-2,8-dione moiety. It is presumed that the parent compound undergoes a double bond shifting along with the formation of the ring with the elimination of the methyl group attached to the –C(=O)OMe group in the methyl 5-vinyl-2-cycloheptene-1-carboxylate part of the compound (Fig. 8.4).

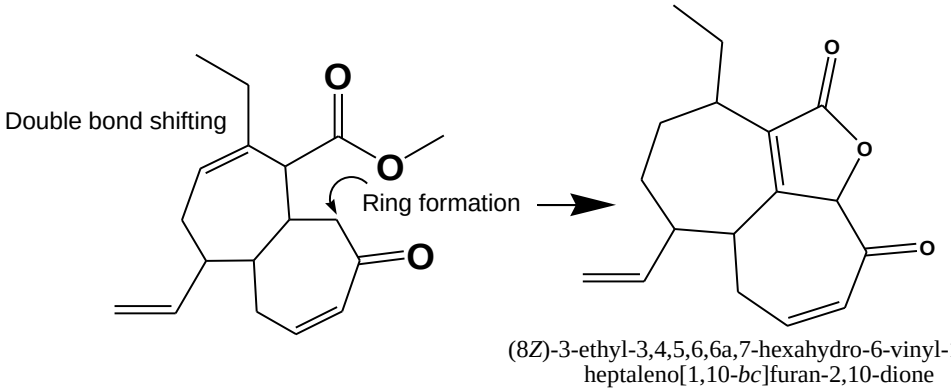


Fig. 8.4. Ring formation of the parent compound methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate to (8Z)-3-Ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione

The spectroscopic characterization of the pure and active compound (KA-36), (8Z)-3-Ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione are discussed in detail.

(8Z)-3-Ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc] furan-2,10-dione: (2Z,7Z)- methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate: Greenish oil; UV (MeOH) λ_{max} (log ε): 292 (3.89), 362 (2.98) nm; TLC (Si gel GF_{254} 15 mm; 1% MeOH/$CHCl_3$, v/v) R_f:0.81; GC R_t:12.22min; IR (KBr, cm^{-1}) ν_{max} 731.29 ρ (C-H), 1376.26 ρ (C-H), 1454.38 δ (C-H alkanes),1652 ν (C=C), 1690.21 ν (C-CO-C), 2854.74 ν (C-H); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 5.83 (m, 1H), 5.37(d, 1H, J=6.5), 4.98(d, 2H, J=6.5), 4.33(m, 1H), 3.69(s, 1H); ^{13}C-NMR ($CDCl_3$) δ 177.37, 162.65, 143.71, 135.25, 125.04, 118.85, 107.84, 55.22, 46.38, 37.42, 32.44, 31.94, 29.37, 26.41, 22.20, 14.12 (details under the Table 8.2); ^{1}H-^{1}H COSY and HMBC data (details under the Table 8.2); (HRESIMS m/e 295.1424 $[M+Na]^+$; D 0.0 amu, calcd for $C_{17}H_{20}O_3Na$, 295.3362).

Compound I (KA-36), a new derivative of the substituted heptalenofurandione, was isolated as greenish oil upon repeated column chromatography using silica gel as adsorbent. There are no significant differences in the ^{1}H-NMR and ^{13}C-NMR spectra of the parent compound, (2Z,7Z)- methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate and (8Z)-3-ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione as indicated in the Table 8.2. An extra –CH proton appeared downfield at δ 3.69 (s, 1H) that signify a possible oxygenation or α, β-unsaturated moiety in its vicinity. The downfield shift of the –CH proton also referred to be a part of the cyclic ring structure. The olefinic bond was found to be shifted from the C3-C4 position to C2-C8 position as explained by the ^{1}H–^{1}H COSY and HMBC experiments (Table 8.2; Fig. 8.5.1). Unlike the parent compound no methylene signal is apparent at δ 2.17 ppm due to the protons bearing 9-C, which also indicate the presence of the furanone ring structure. The DEPT showed characteristic –CH- peak in the olefinic region at δ 143.71 ppm, which exhibited strong ^{1}H–^{1}H COSY spectrum, and coupled with the protons at δ 2.36 ppm belonging to the carbon atom at δ 37.42 ppm. This indicates

that the olefinic bond is situated internal to the ring system. The ^{13}C NMR spectrum of the purified compound in combination with DEPT experiments indicated the occurrence of 17 carbon atoms in the molecule including four quaternary carbons, four –CH, five $–CH_2$, and one $–CH_3$ carbons. The ^{13}C NMR spectrum of the purified compound displayed two quaternary carbon (δ 177.37, 162.65) atoms bearing the carbonyl groups. The side chain ($-CH_2CH_3$) is substituted at C-3 (δ 37.42), and its upfield shift as compared to the parent compound clearly spell out the olefinic bond shifting in the compound to a different location. The cyclic rings were confirmed by the strong $^1H–^1H$ COSY signals of $–CH_2$ and -CH protons supported HSQC and HMBC signals as detailed under Table 8.2. The proton at 3-H (δ 2.36) exhibited strong $^1H–^1H$ COSY correlation with the proton at δ 1.26 ppm (assigned to be 4-H), which in turn exhibited $^1H–^1H$ COSY correlations between H-4/H-5,H-6/H-7 (Table 8.2). These correlations assigned the octahydroheptalene ring system as in parent compound. In the $^1H–^1H$ COSY spectrum, couplings were apparent between H-13/H-12; H-5/H-6/H-7 which support the presence of octahydroheptalene skeleton. The $^1H–^1H$ COSY correlations between H-6 (δ 2.05)/H-14 (δ 5.83) supports the presence of the terminal vinylic moiety at the cycloheptene ring system. The proton and carbon connectivity deduced from HSQC and HMBC experiments confirmed the octahydroheptalene framework. Placement of the ethyl group at C-3 of the cycloheptene ring was confirmed by the upfield shift of H-17 (δ 0.9) in the compound as compared to the baseline value in the HMBC spectrum. The geometric isomerisms of the olefinic protons (Table 8.2) have been established by the 1H coupling constants suitable for the geometries (Z form). NOE couplings were observed between Hα-3 (δ 2.36)/Hα-7 (δ 4.10) thus indicating that these groups must be equatorial and on the α-side of the molecule. NOE correlations between Hβ-6 (δ 2.05)/Hβ-9 (δ 3.69) indicated the close proximity of these groups and their β-disposition. The methine proton (-CH) at C-7 group (δ 4.10) did not exhibit NOE interactions with H-6 (δ 2.05), which is at the β-face of the molecule, thereby indicating that H-7 is at the equatorial disposition.

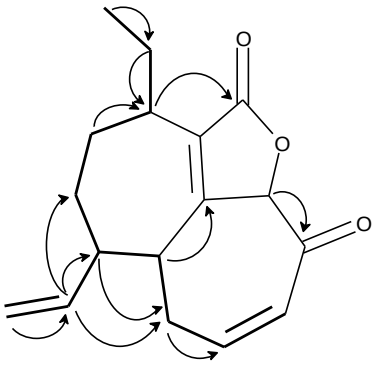
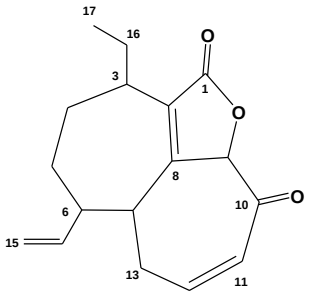


Fig. 8.5.1 . ^{1}H–^{1}H COSY and HMBC correlations of (8Z)-3-ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione. The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

As compared to the parent compound (2Z,7Z)-methyl-2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate, the strong bending vibration bands near 1736 cm^{-1} denotes the substituted furanone absorption. The olefinic (C=C) groups have been symbolized by the absorption bands at 1652 and 1056 cm^{-1}. There were no notable differences in the IR spectra between the compounds. The ultraviolet absorbances at λ_{max} (log e) 292 (3.89), 362 (2.98) nm shifted as compared to the parent compound, thus indicating the presence of more than one conjugated system in the compound. Its mass spectrum exhibited a molecular ion peak at m/e 272 (HRESIMS m/e 295.14 $[M+Na]^{+}$; D 0.0 amu, calcd for $C_{17}H_{20}O_3Na$, 295.3362), which in combination with its ^{1}H and ^{13}C NMR data (Table 8.2) indicated the elemental composition of $C_{17}H_{20}O_3$ as (8Z)-3-ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione with eight degrees of unsaturation. Seven degree of unsaturation from five double bonds of methyl 2-ethyl-octahydro-9-oxo-5-vinylheptalene-1-carboxylate moiety, and the two degree of unsaturation from the two ring system of octahydroheptalene. The additional degree of unsaturation was due to the furanone ring system. The mass fragmentation pattern shows similarity between the parent compound and (8Z)-3-ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione. The fragments (3Z, 8Z)-9-ethyl-5, 5a, 6, 7, 10, 10a-hexahydro-6-vinylheptalen-2(1H)-one at m/e 230.34 ($C_{16}H_{22}O^{\bullet+}$), m/e 112.21 ($C_8H_{16}^{\bullet+}$), m/e 110.15 ($C_7H_{10}O^{\bullet+}$) are similar to that noted in the parent compound. Appearance of the fragment at m/e 166.30 indicates the presence of dodecahydroheptalene moiety ($C_{12}H_{22}^{\bullet+}$), resulted from the reductive intra-molecular rearrangement of ethyl-hexahydro-6-vinylheptalen-2(1H)-one. The base peak was found to be as cyclohexanone ($C_6H_{10}O^{\bullet+}$, m/e: 98.14).

The activity of the compound appeared to be due to the presence of –C(=O)OMe and vinyl groups as in methyl 5-vinyl-2-cycloheptene-1-carboxylate part of the compound. The multiple electronegative centres of the compound and electron delocalization due to the tautomeric conversion of the cycloheptenone and furanone rings of the compound to their enol forms appeared to serve as the potential electronegative centres of the compound resulting in higher antioxidative potential.

Table 8.2 NMR spectroscopic data of (8Z)-3-ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione in $CDCl_3$.[a]



Carbon no.	^{13}C NMR (DEPT)	H	δ^{1}H NMR (int., mult., J in Hz)[b]	^{1}H-^{1}H COSY	HMBC (^{1}H-^{13}C)
1	177.37				
2	143.71				
3	37.42	3H	2.36(m,1H)	H-4,H-16	C-1
4	26.41	4H	1.26(m,2H)	H-5	C-3
5	22.20	5H	1.39(m,2H)	H-6	
6	31.94	6H	2.05(m,1H)	H-7,H-14	C-13
7	46.38	7H	4.10(m,1H)	H-13	C-8
8	140.15				
9	55.22	9H	3.69(s,1H)		C-10
10	162.65				
11	125.04	11H	5.37(d,1H,J=6.5)	H-12	
12	107.84	12H	4.33(m,1H)	H-13	
13	32.44	13H	1.97(m,2H)	H-7,H-12	C-12
14	135.25	14H	5.83(m,1H)	H-15	C-5,6,13
15	118.85	15H	4.98(d,2H,J=6.5)	H-14	C-14
16	29.37	16H	1.55(m,2H)	H-3	C-3
17	14.12	17H	0.9(t,3H,J=13.9)	H-16	C-16

[a] NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers.

[b] Values in ppm, multiplicity and coupling constants (J¼ Hz) are indicated in parentheses. Assignments were made with the aid of the ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY experiments.

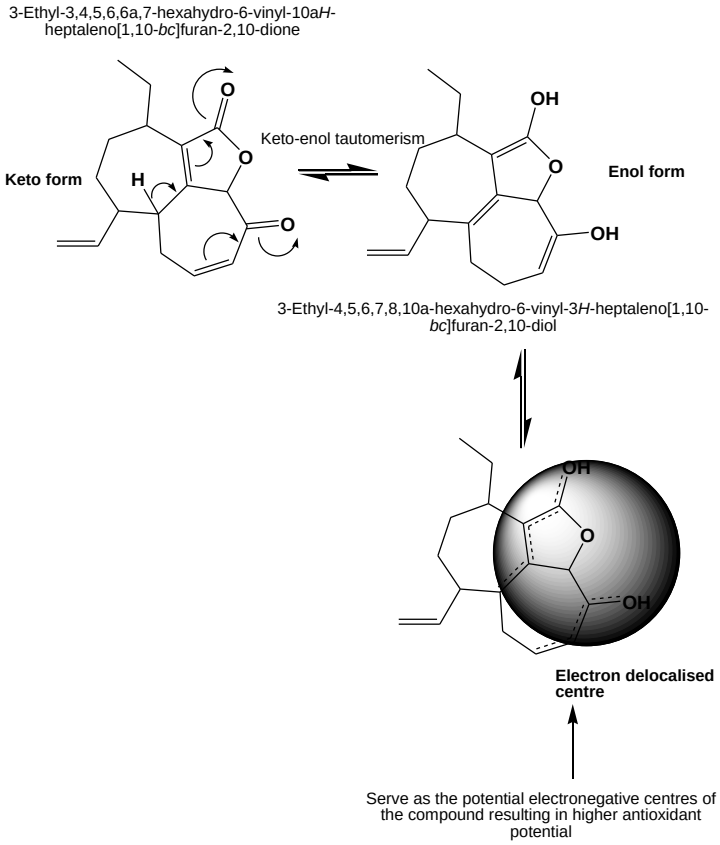


Fig. 8.5.2. Keto enol tautomerism of 3-ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione leading to the formation of 3-ethyl-4,5,6,7,8,10a-hexahydro-6-vinyl-3H-heptaleno[1,10-bc]furan-2,10-diol

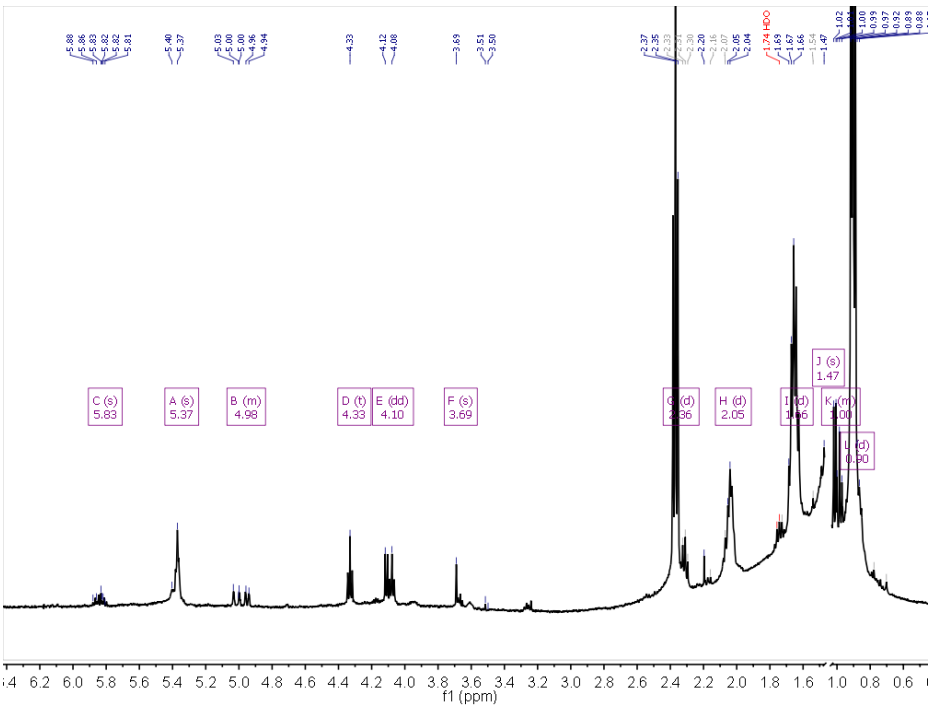


Fig. 8.5.3. Proton NMR spectrum of (8Z)-3-ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione

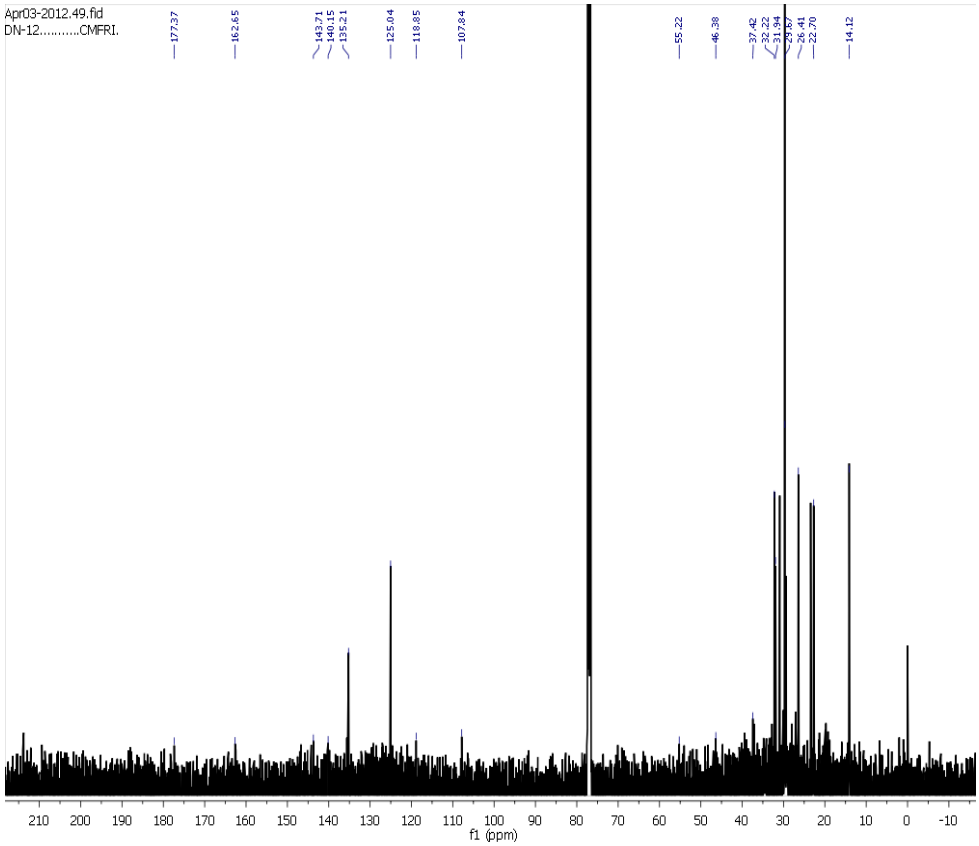


Fig. 8.5.4. ^{13}C NMR spectrum of (8Z)-3-ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione

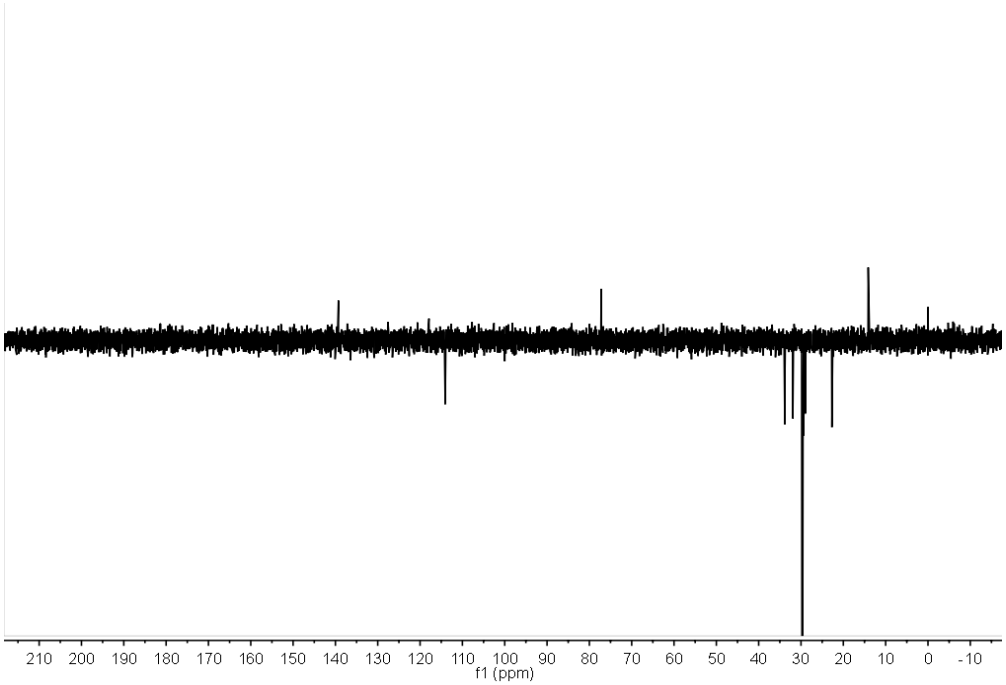


Fig. 8.5.5. DEPT spectrum of (8Z)-3-ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione

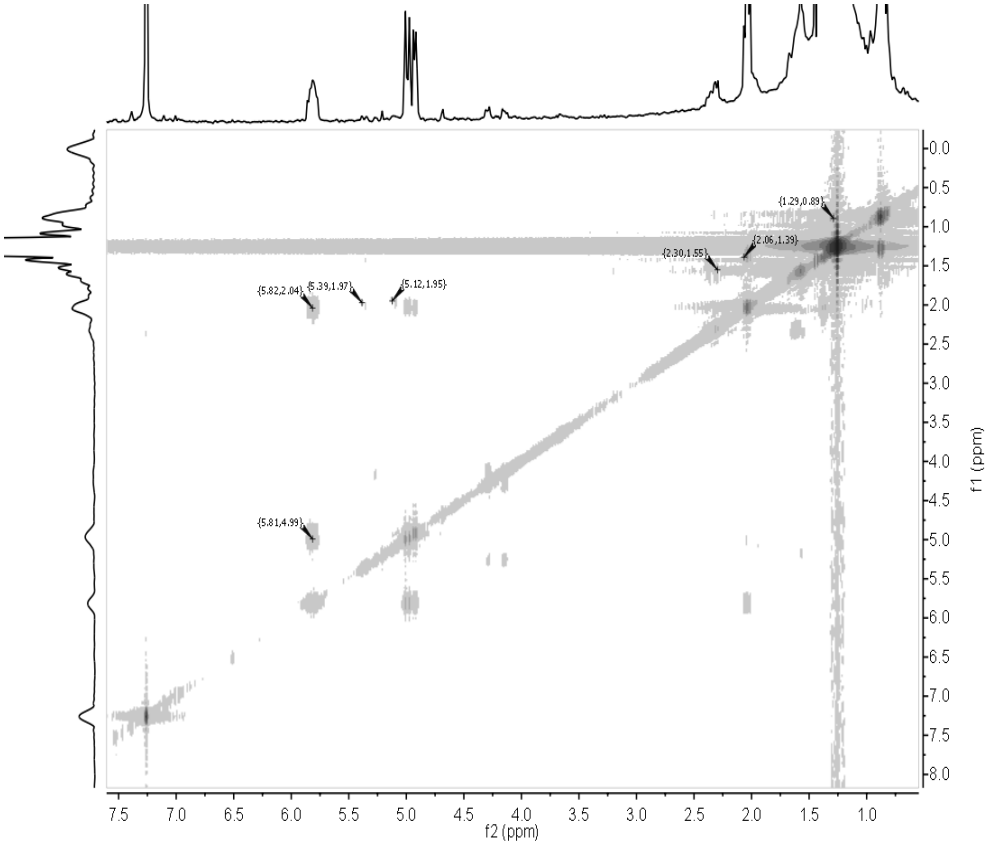


Fig. 8.5.6 ^{1}H–^{1}H COSY spectrum of (8Z)-3-ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione

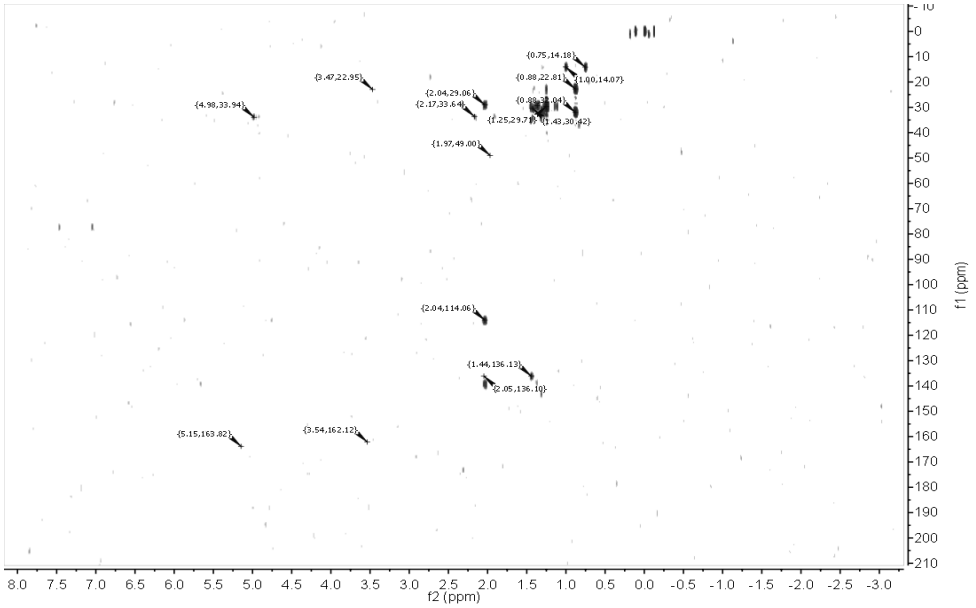


Fig. 8.5.7. HMBC spectrum of (8Z)-3-ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione

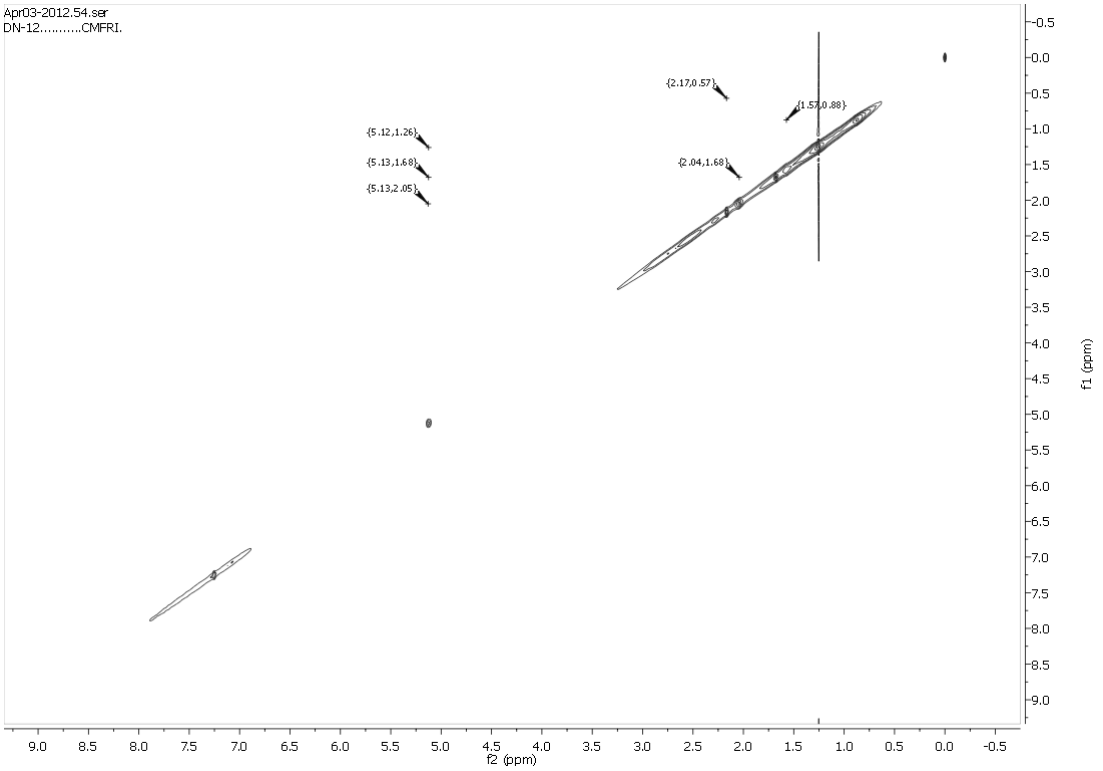


Fig. 8.5.8. NOE spectrum of (8Z)-3-ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione

The furandione derivatives isolated from the natural sources were found to possess potential bioactive properties. 5-hydroxy-3, 4, 7-triphenyl-2, 6-benzo furandione has been isolated from *Peniophora sanguinea* was reported to possess antibacterial properties (Hartl *et al.*, 1998). Tetrahydro-3, 4-furandione was found to possess pluralities of biological activities (Kendall & Hajos 1960). 7-Methoxy-2, 3-dimethylbenzofuran-5-ol isolated from the fungus *Malbranchea cinnamomea* HKI 0286 was found to possess potential antioxidative properties and inhibitor of peroxidase as key enzyme of oxygen radical generation (Schlegel *et al.*, 2003). 5-Hydroxy-3, 4, 7-triphenyl-2, 6-benzofurandione, was isolated from *Peniophora sanguinea*, and was found to inhibit xanthine oxidase (Hartl *et al.*, 1998).

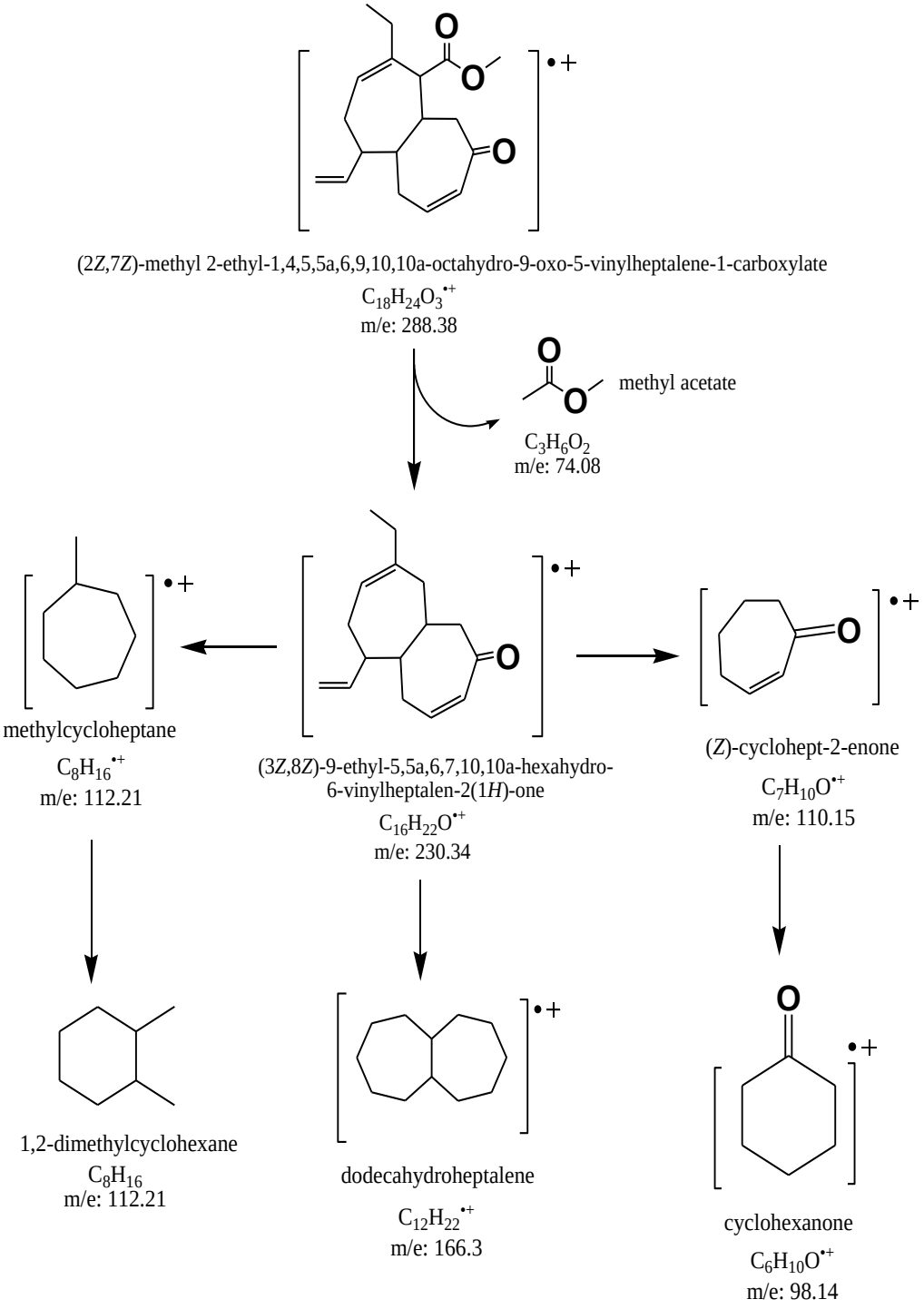
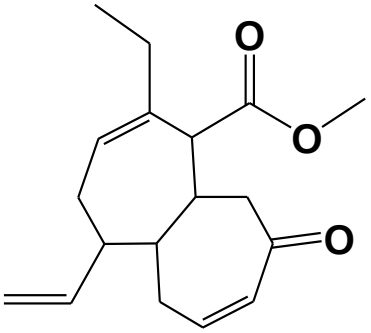


Fig. 8.5.9. Mass fragmentation pattern of (2Z, 7Z) - methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate.

8.2.1.2. Structural Characterization of Compound II (KA-37)

COMPOUND II (KA-37)

(2Z, 7Z) - methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1- heptalenecarboxylate



Sample yield	61.5 mg (5.1 %*)
Physical description	Yellow powder
Molecular formula	$C_{18}H_{24}O_3$
Molecular weight	288.3956

* yield with respect to EtOAc fraction

The spectroscopic characterization of the pure and active compound (KA-37), (2Z,7Z)- Methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate are discussed in detail below.

(2Z,7Z)- Methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate: Yellow powder; UV (MeOH) λ_{max} (log ε): 286 (3.24), 325 (2.89) nm TLC (Si gel GF_{254} 15 mm; 20 % EtOAc/*n*-hexane, v/v) R_f: 0.54; GC R_t:5.40min.; IR (KBr, cm^{-1}) ν_{max} 728.29 ρ (C-H), 1056 ν (C-O), 1376.26 ρ (C-H), 1454.38 δ (C-H alkanes), 1585.17 ν (C-C in ring), 1642.24 ν (C=C), 1690.21 ν (C-CO-C), 1736.96 (C=O), 2854.74 ν (C-H), 2955.04 ν (C-H); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.81(m, 1H), 5.35(d, 1H, J=13.4), 5.02 (t, 1H), 4.96(d, 2H, J=6.5), 3.69 (s, 3H), 2.30 (d, 1H, J=6.25), 0.89 (t, 3H); ^{13}C-NMR ($CDCl_3$) 206.91, 174.37, 151.55, 147.07, 129.74 (details under the Table 8.3). ^{1}H-^{1}H-COSY and HMBC data (details under the Table 8.3); HRESIMS m/e:288.6242($C_{18}H_{24}O_3$): found 288.6242 (calcd. for $C_{18}H_{24}O_3$ 288.1462).

(2Z,7Z)-Methyl-2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate, a new derivative of the substituted heptalenecarboxylate was

isolated as yellowish powder substance upon repeated chromatography over silica columns. The ^{1}H-NMR in conjugation with ^{13}C-NMR recorded the presence of two methyl signals at δ 0.89 and δ 3.69. The methyl group at δ 3.69 shifted downfield due to the electronegative group bearing oxygen atom at the α-position with respect to the deshielded methyl group. The methylene group protons at δ 1.72 and δ 2.17 are assigned to be at 13-C and 9-C, respectively of the cycloheptene ring structure and the downfield shift (about δ 0.45) is due to the presence of the conjugated cycloheptenone moiety. The $–CH_2$- protons appeared at δ 1.99 ppm are due to the presence of the cycloheptene and have been assigned to be present at the C-5 position of the ring structure. The –CH proton at δ 2.04 is shifted downfield due to the presence of –C=C- group at its α-position. The carboxyl ester group at the C1 position of the cycloheptene ring resulted in strong deshielding of the –CH- proton at δ 2.30 ppm and therefore, has been assigned to be present at the C2 position of the cycloheptene ring structure. The methine proton (gives triplet of doublet) at δ 2.30 is characteristic of the junction point of the cycloheptene ring (C-2) with that of the side chain methyl acetate moiety. The DEPT experiment showed characteristic -CH_2 peak in the olefinic region at δ 114.05 ppm, which exhibited strong ^{1}H–^{1}H COSY spectrum, and coupled with the protons at δ 5.81 ppm belonging to the carbon atom at δ 139.26. This indicates that the olefinic bond is situated external to the ring system. The ^{13}C-NMR spectrum of the purified compound in combination with DEPT experiments indicated the occurrence of 18 carbon atoms in the molecule including 3 quaternary carbons, eight –CH, five $–CH_2$ and two $–CH_3$ carbons assigned. ^{13}C-NMR showed characteristic olefinic signals at δ 147.07, 129.74 (assigned to be at C-3-C-4), δ 151.55, 100.1 (assigned to be at C-11 & C-12) and δ 139.26, 114.05 (assigned to be at C-14& C-15) indicate the presence of three double bonds. The ^{13}C NMR spectrum of the purified compound displayed two quaternary carbon (δ 147.07, 206.91) atoms bearing the olefinic and carbonyl groups. The side chain (-Et) is substituted at C-3 (δ 147.07). The cyclic rings were confirmed by the strong ^{1}H–^{1}H COSY signals of methylene and methine protons supported HSQC and HMBC signals as detailed under Table 8.3. The carboxyl carbon attached to ring at C-2 has been confirmed by detailed HMBC experiment and mass spectral analyses,

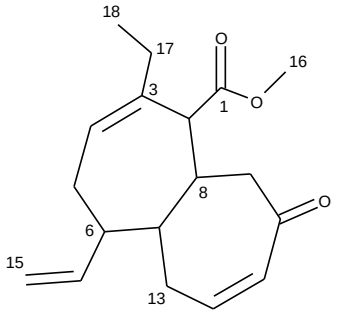
whereas one methoxy carbon at C-16 (δ 51.43) showed HSQC correlation with 16-H (δ 3.69, s) situated on C-1 by HMBC. The proton at 2-H (δ 2.30) exhibited strong $^{1}H–^{1}H$ COSY correlation with the proton at δ 1.64 ppm (assigned to be 8-H). The methylene and methine protons in the octahydroheptalene ring system were connected by strong $^{1}H–^{1}H$ COSY correlations between 2-H/8H, 8-H/9-H, 8-H/7-H, 6-H/7-H, 6-H/5-H. In the $^{1}H–^{1}H$ COSY spectrum, couplings were apparent between H-8/H-9; H-13/H-12; H-5/H-6/H-7 and H-2/H-8 which support the presence of octahydroheptalene skeleton. The point of cyclization was indicated by the low-field shift of H-8 and H-7 at δ 1.64 and δ 1.35, respectively which has been coupled to the H-13/9 methylene protons at δ 1.72, 2.17 (Table 8.3) which also gives clear $^{1}H–^{1}H$ COSY correlation. Also, the $^{1}H–^{1}H$ COSY correlations between H-6 (δ 2.04)/H-14 (δ 5.81) supports the presence of the terminal vinylic moiety at the cycloheptene ring system. The proton and carbon connectivity deduced from HSQC and HMBC experiments confirmed the octahydroheptalene framework. The H–H and C–H connectivity apparent in the $^{1}H–^{1}H$ COSY and HMBC spectra respectively indicate that two of the seven degrees of unsaturation were due to the two ring system of octahydroheptalene ring framework. In the HMBC spectrum, it was observed that H-2/C-1; H-5/C-6; H-6/C-14, 15; and H-17/C-3 were correlated with each other (Table 8.3). In addition, a methine proton (H-6, δ 2.04) was coupled to the olefinic tertiary carbon (C-14, δ 139.26) and methylene H-5 (δ 1.99) with C-6 (δ 33.8). This indicated that H-6 was connected to the olefinic tertiary carbon atom. The proton at C-16 appeared to demonstrate long range HMBC correlation (Fig. 8.6.1) with ester carbonyl carbon at δ 174.37 (C-1) and the methine carbon at δ 34.13 (C-2). This established the side chain methyl acetate linked to the cycloheptene moiety at C-2. The HMBC spectrum of the purified compound also revealed connections of the proton (H-2, δ 2.30) to the methine carbon at δ 34.13. Placement of the ethyl group at C-3 of the cycloheptene ring was confirmed by the downfield shift of H-17 (δ 1.28) in the compound as compared to the baseline value and also by the long-range correlation of H-17 to the olefinic carbon at δ 129.74 (C-4) in the HMBC spectrum. The geometric isomerisms of the olefinic protons (Table 8.3) have been established by the 1H coupling constants suitable for the geometries (Z form). NOE couplings

were observed between Hα-2 (δ 2.30)/Hα-6 (δ 2.04)/Hα-8 (δ 1.64) thus indicating that these groups must be equatorial and on the α-side of the molecule. Therefore, the C-1 carboxyl group is axial and β-oriented. NOE correlations between Hβ-7(δ 1.35)/Hβ-4 indicated the close proximity of these groups and their β-disposition. The methine proton at C-8 group (δ 1.64) did not exhibit NOE interactions with H-7 (δ 1.35), which is at the β-face of the molecule, thereby indicating that H-8 is at the equatorial disposition. The NOESY spectra experiment showed spatial correlation between 4-H (δ 5.02), 18-H (δ 0.89), and 6-H (δ 2.04). NOESY correlation between 16-H (δ 3.69) and 2-H (δ 2.30) indicates that 18-H is at close proximity to the carboxylate ester.

The IR absorption band (in MeOH) near 1736 cm^{-1} denotes the ester carbonyl absorption. The olefinic (C=C) groups have been symbolized by the absorption band at 1642 cm^{-1}. The carbons attached to oxygen showed C-O stretching near 1056 cm^{-1}. The ultraviolet absorbances at λ_{max} (log e) 286 (3.24), 325 (2.89) nm were assigned to a chromophore with unsaturation. Its mass spectrum exhibited a molecular ion peak at m/e 288 (HRESIMS m/e 311.2982 $[M+Na]^+$; D 0.0 amu, calcd for ($C_{18}H_{24}O_3Na$): 311.1462), which in combination with its ^1H and ^{13}C NMR data (Table 8.3) indicated the elemental composition of $C_{18}H_{24}O_3$ as (2Z,7Z)-methyl 2-ethyl-1,4,5,5a,6,9,10,10a-octahydro-9-oxo-5-vinylheptalene-1-carboxylate with seven degrees of unsaturation. Seven degree of unsaturation from five double bonds of methyl 2-ethyl-octahydro-9-oxo-5-vinylheptalene-1-carboxylate moiety, and the two degree of unsaturation from the two ring system of octahydroheptalene. The molecular ion peak at m/e 288.38 ($C_{18}H_{24}O_3^{\bullet+}$) appeared to undergo elimination of methyl acetate ($C_3H_6O_2$, m/e: 74.08) to yield (3Z,8Z)-9-ethyl-5,5a,6,7,10,10a-hexahydro-6-vinylheptalen-2(1H)-one at m/e 230.34 ($C_{16}H_{22}O^{\bullet+}$), which undergoes intramolecular rearrangement to afford the fragments with m/e 112.21 ($C_8H_{16}^{\bullet+}$) assigned to be as methylcycloheptane, and m/e 110.15 ($C_7H_{10}O^{\bullet+}$) assigned to be as (Z)-cyclohept-2-enone. Appearance of the fragment at m/e 166.30 indicates the presence of dodecahydroheptalene moiety ($C_{12}H_{22}^{\bullet+}$), resulted from the reductive intramolecular rearrangement of ethyl-hexahydro-6-vinylheptalen-2(1H)-one (Fig. 8.6.2). The base peak was found to be as cyclohexanone ($C_6H_{10}O^{\bullet+}$, m/e: 98.14),

whereas peaks of good intensity appeared at m/e 112.21 (C_8H_{16}) apparently due to 1, 2-dimethylcyclohexane moiety.

Table 8.3 NMR spectroscopic data of (2Z,7Z)- methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate in $CDCl_3$.[a]



Carbon no.	^{13}C NMR (DEPT)	H	δ^{1}H NMR (int., mult., J in Hz)[b]	^{1}H-^{1}H COSY	HMBC (^{1}H-^{13}C)
1	174.37				
2	34.13	2H	2.30(d,1H,J=6.25)	H-8	C-1
3	147.07				
4	129.74	4H	5.02(t,1H)	H-5	C-6
5	29.68	5H	1.99(m,2H)	H-6	C-6
6	33.8	6H	2.04(m,1H)	H-7,H-14	C-14,15
7	31.93	7H	1.35(m,1H)	H-8,H-13	
8	24.81	8H	1.64(m,1H)	H-9	C-9
9	30.9	9H	2.17(d,2H,J=6.25)		C-10
10	206.91				
11	151.55	11H	5.35(d,1H,J=13.4)		
12	100.1	12H	4.31(m,1H)	H-13	C-13
13	29.82	13H	1.72(m,2H)	H-7	C-7
14	139.26	14H	5.81(m,1H)	H-15	
15	114.05	15H	4.96(d,2H,J=6.5)	H-14	
16	51.43	16H	3.69(s,3H)		C-1
17	22.76	17H	1.28(q,2H)	H-18	C-3
18	14.07	18H	0.89(t,3H)		C-3

[a] NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers.
[b]Values in ppm, multiplicity and coupling constants (J¼ Hz) are indicated in parentheses. Assignments were made with the aid of the ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY experiments.

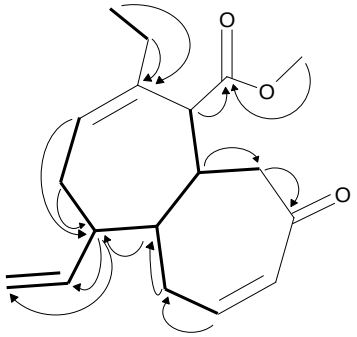
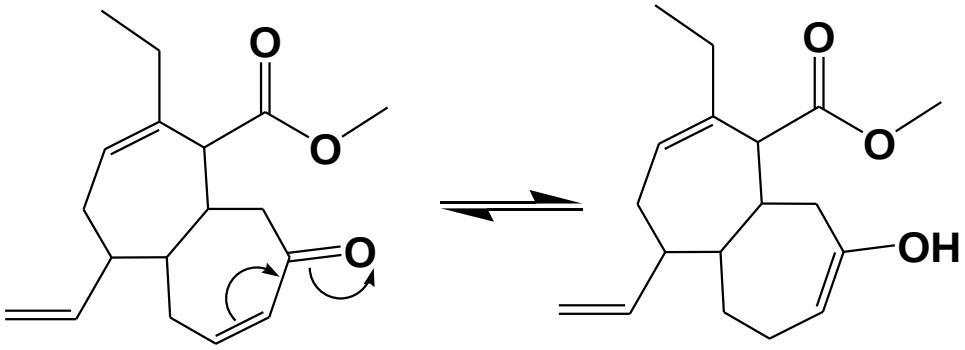


Fig. 8.6.1 ^{1}H–^{1}H COSY and HMBC correlations of (2Z,7Z)- methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate. The key 1H-1H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

(2Z, 7Z)- Methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate nucleus as substituted octahydroheptalenone with two fused rings have been confirmed by detailed NMR and mass spectroscopic techniques, The stereochemistry of the olefinic groups have been confirmed as Z- type by calculating the J-values and detailed NOE experiments.



Methyl 2-ethyl-9-hydroxy-5-vinyl-1,4,5,5a,6,7,10,10a-octahydro-1-heptalenecarboxylate

Fig. 8.6.2. Keto-enol tautomeric interconversion of (2Z, 7Z) - methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate

The activity of the compound appeared to be due to the presence of –C(=O)OMe and vinyl groups as in methyl 5-vinyl-2-cycloheptene-1-carboxylate part of the compound. The multiple electronegative centres of the compound and electron delocalization due to the tautomeric conversion of the cycloheptenone ring of the compound to cycloheptenol appeared to serve as the potential electronegative centre of the compound.

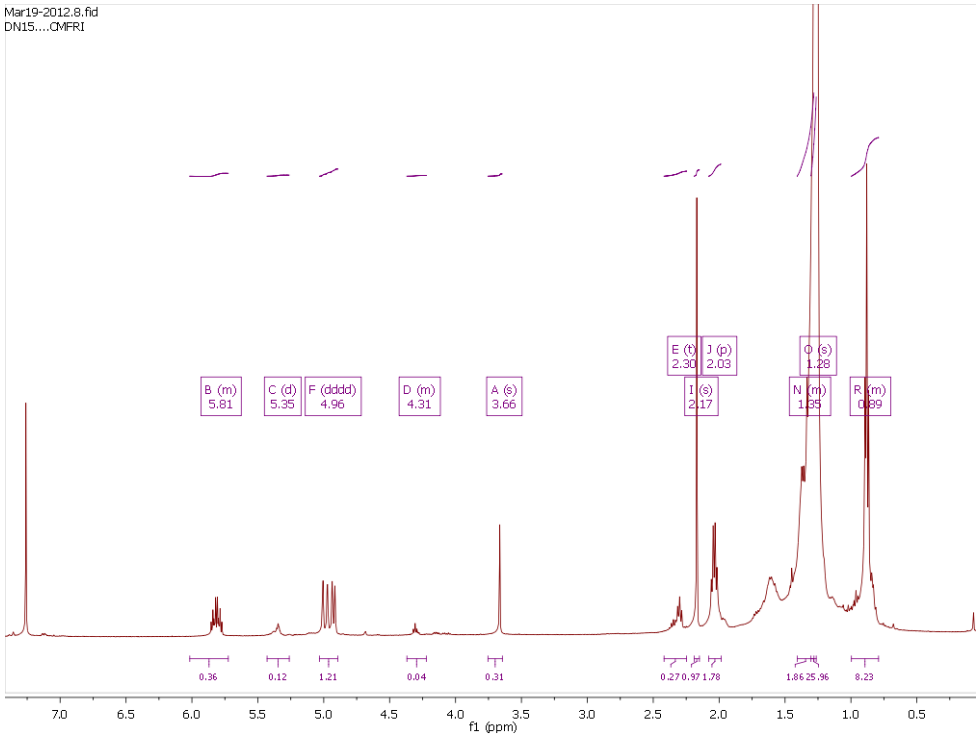


Fig. 8.6.3 Proton NMR spectrum of (2Z,7Z)- methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate

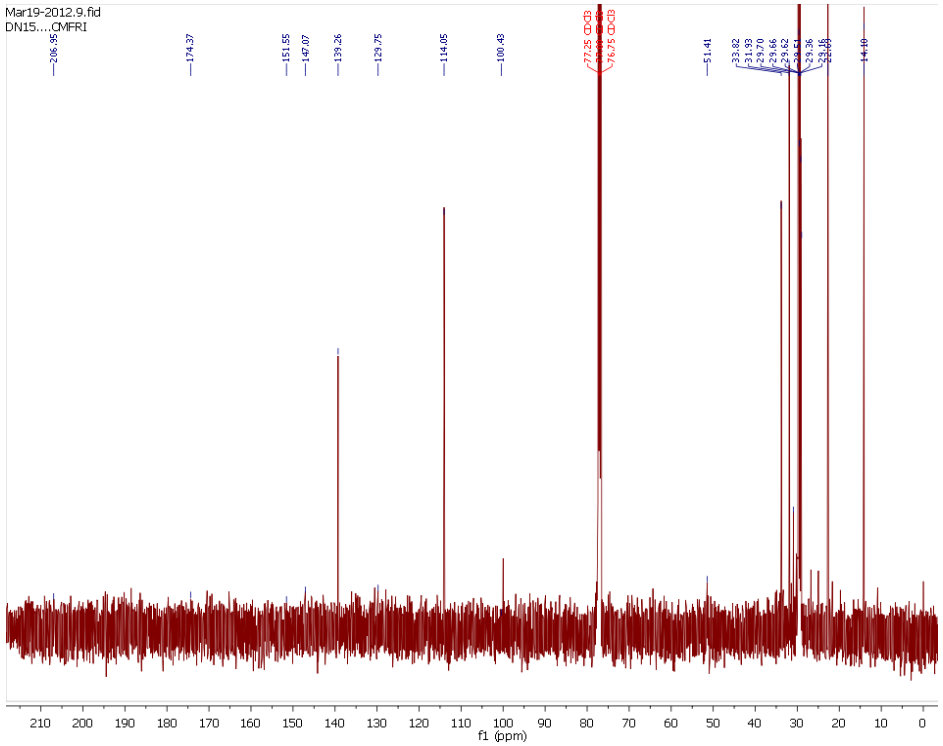


Fig. 8.6.4. ^{13}C NMR spectrum of (2Z,7Z)- methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate

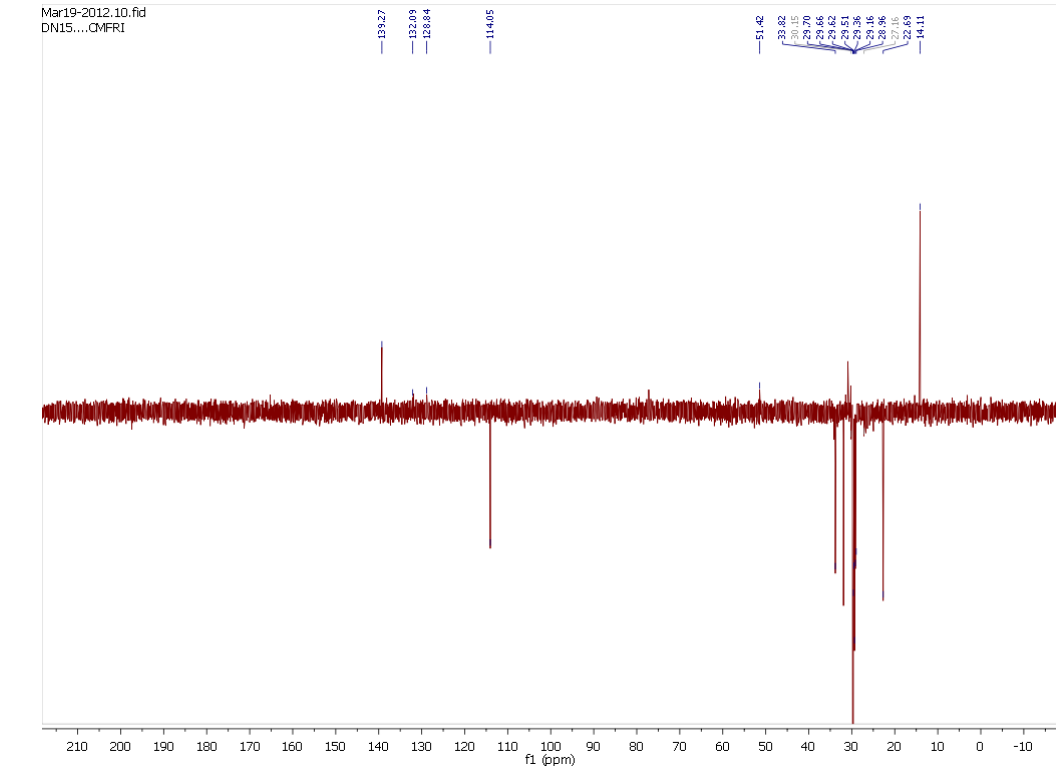


Fig. 8.6.5. DEPT spectrum of (2Z,7Z)- methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate

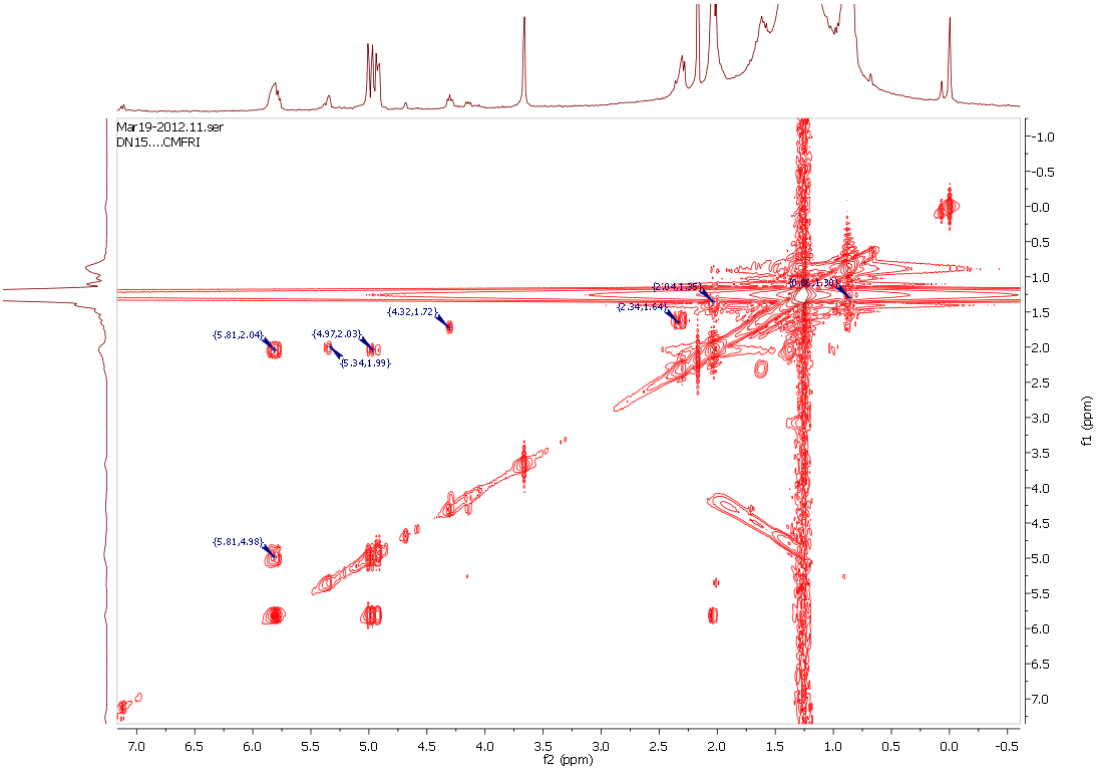


Fig. 8.6.6 ^{1}H–^{1}H COSY spectrum of (2Z,7Z)- methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate

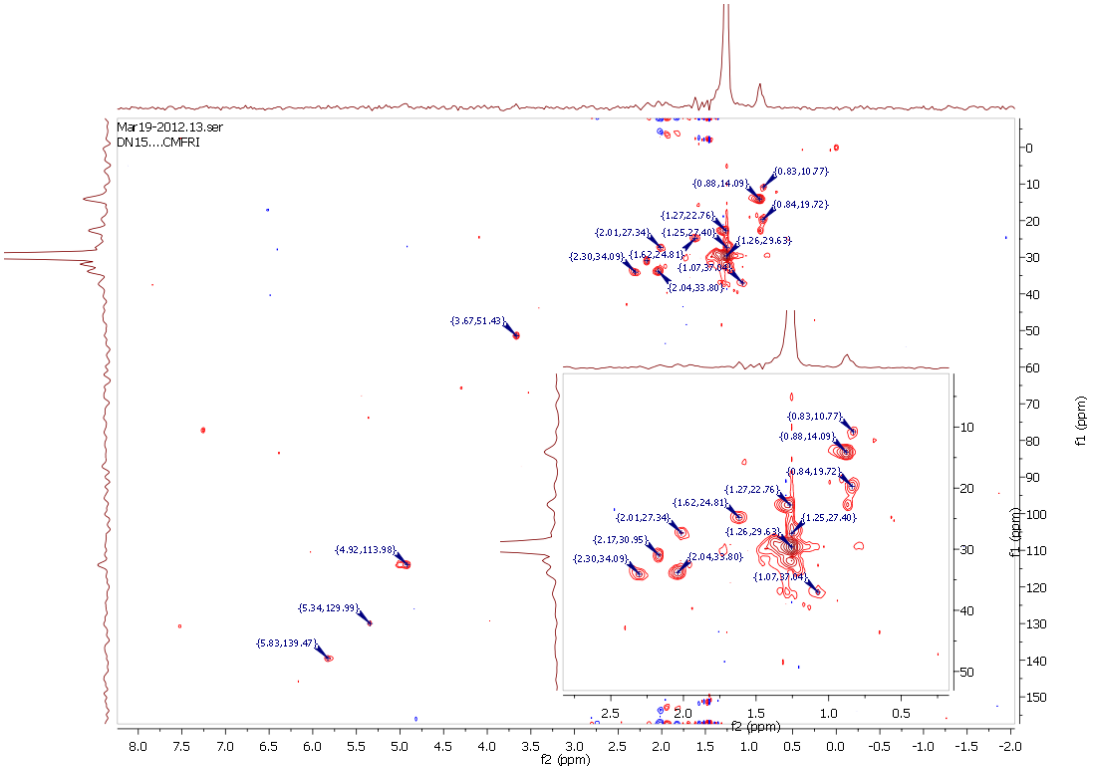


Fig. 8.6.7. HSQC spectrum of (2Z,7Z)- methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate

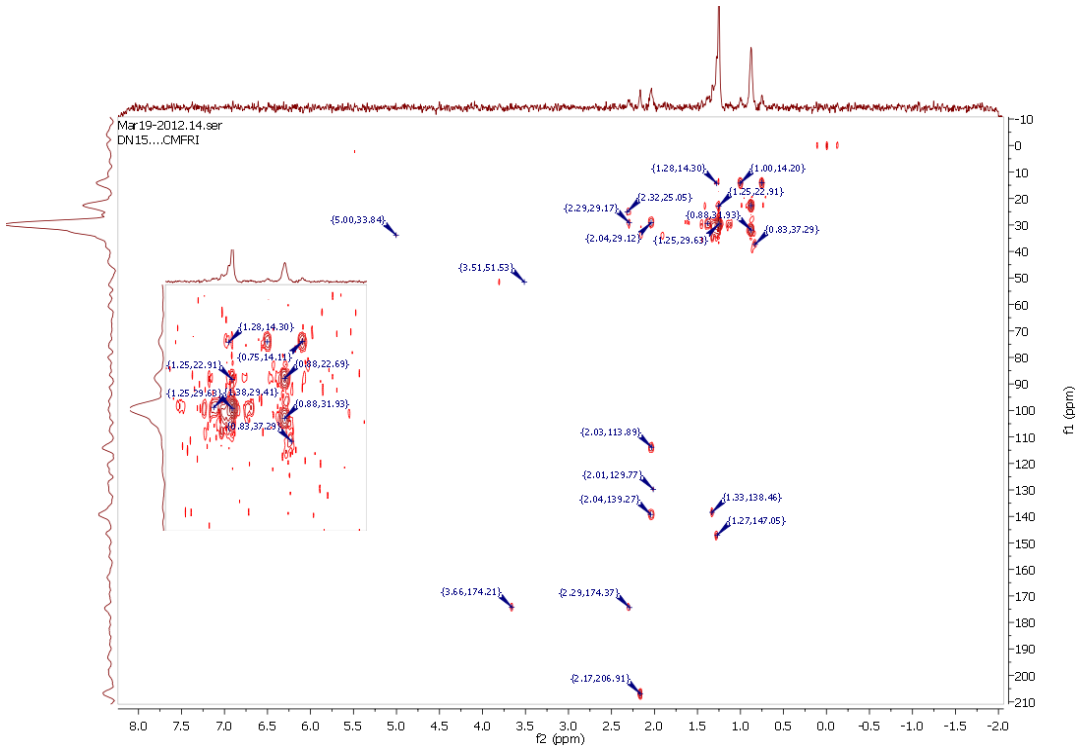


Fig. 8.6.8. HMBC spectrum of (2Z,7Z)- methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate

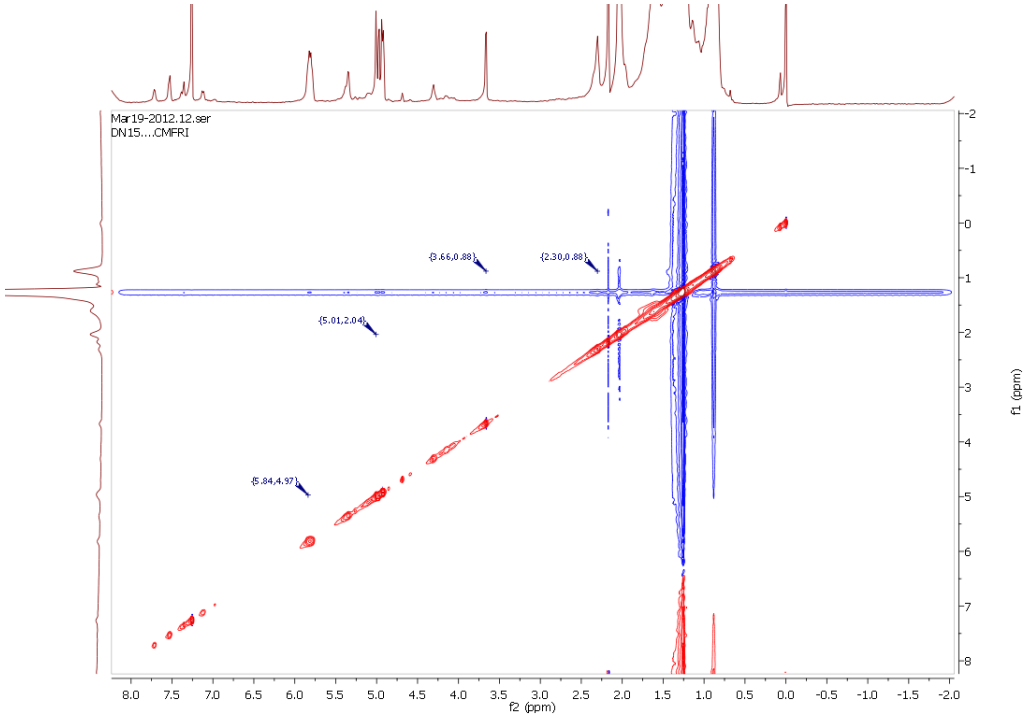


Fig. 8.6.9. NOE spectrum of (2Z,7Z)- methyl 2-ethyl-9-oxo-5-vinyl-1,4,5,5a,6,9,10,10a-octahydro-1-heptalenecarboxylate

The heptalene derivatives were not been reported from the marine organisms although there are reports of the synthetic derivatives of 1,5,6,8,10 - pentamethylheptalene - 4 - carboxaldehyde and methyl 4 - formyl - 1,6,8,10 - tetramethylheptalene - 5 - carboxylate (Landmesser *et al.*, 2013). The donor/acceptor mechanisms of the substituted heptalenes have been discussed in the earlier literature. Methyl heptalene carboxylates of type A and B with substituents have been reported and their structures have been characterized by detailed spectroscopic techniques (Sarah *et al.*, 2013).

8.2.2. Secondary Metabolites from *Hypnea musciformis*

The yield, antioxidant activities of each column/P-TLC/P-HPLC fractions are given in Table 8.4. The R_f of all the p-TLC fractions are also shown in Table 8.4. Among the column fractions obtained from the EtOAc fraction of the MeOH extract, the fractions HM-1 (721.6 mg), HM-2 (514.3 mg), HM-4 (1246.3 mg) and HM-7 (1523.4 mg) exhibited significantly higher antioxidant activity with respect to

scavenge DPPH radicals (76.2, 80.2, 64.4 & 76.1 %, respectively) ($p<0.05$). Among these fractions, both HM-1 and HM-2 showed significantly higher Fe^{2+} ion chelating activity (41.3 & 22.2 %, respectively). HM-4 exhibited maximum lipid peroxidation inhibitory activity (0.26 MDAEQ/kg) followed by HM-2 (0.61 MDAEQ/kg) and HM-5 (0.64 MDAEQ/kg).

HM-1 was further purified using p-TLC (20% EtOAc:*n*-hexane) to get a pure active compound, HM-9 (Rf 0.25) with low yield (4.7 mg) along with other fractions HM-10, 11 and 12. HM-11 on further chromatography using p-TLC (20% EtOAc:*n*-hexane) obtained HM-13, 14 and 15. Though HM-14 was found to be pure compound the yield was low (7 mg).

The fraction, HM-2 was purified using CC (*n*-hexane/EtOAc/MeOH) to obtain 8 fractions, HM-16 to HM-23. The sub-fraction, HM-20 which found to have 70.1 % DPPH radical scavenging activity and 28.1 % Fe^{2+} ion chelating activity was further purified using CC (*n*-hexane/EtOAc) to obtain an active pure compound (HM-25) with 46.5 % DPPH radical scavenging activity. However the yield was found to be low for HM-25 (3.1 mg).

Among the first column fractions, HM-4 which exhibited high radical scavenging ability as realized from its DPPH radical scavenging ability (64.4 %), lipid peroxidation inhibitory activity (0.26 MDAEQ/kg) was further chromatographed using CC (*n*-hexane/EtOAc/MeOH) to obtain 8 fractions HM-29 to HM-36. Among these nine fractions, HM-31(369 mg) and HM-34(202.1 mg) showed significantly higher DPPH radical scavenging activity (76.1 & 48.3 %, respectively). Interestingly, HM-31 showed best lipid peroxidation inhibitory ability (0.04 MDAEQ/kg). HM-31 was further chromatographed over normal silica p-TLC in 20%EtOAc:*n*-hexane to obtain 6 fractions (HM-37 to HM-42) in which HM-37 and HM-42 showed significantly higher DPPH radical scavenging activity (> 48 %) and significantly higher Fe^{2+} ion chelating activity (> 10 %). HM-37 further yielded HM-43 and HM-44 on chromatography through p-TLC. HM-44 though observed to be pure with high radical scavenging activity (60.1 %) was obtained in low amount (4.25 mg). However, HM-42, which resulted three fractions (HM-45, 46 & 47) on purification using p-TLC yielded a pure

active compound (HM-45, Compound 2). At the same time, HM-34 on further purification resulted in three fractions HM-48, 49 & 50. Among these HM-50 (Compound 2, 81.4 mg) was found to be pure, well active with high radical scavenging activity (56.2 %) and Fe^{2+} ion chelating activity (21.1 %).

HM-7 on subsequent purification using CC ($CHCl_3$: MeOH) resulted in five fractions and the most active fraction, HM-55 (radical scavenging activity – 72.2 % and lipid peroxidation inhibitory activity – 0.14 MDAEQ/kg) was further chromatographed over p-TLC (40%EtOAc:*n*-hexane) to obtain three fractions with HM-57 as pure compound (Rf 0.5). However, the yield obtained for HM-57 was only 10.9 mg.

Table 8.4 DPPH radical scavenging activity, lipid peroxidation inhibitory activity (TBARS formation inhibitory activity) and Fe^{2+} ion chelating activity (%) of the fractions obtained by the purification of *Hypnea musciformis*

	Yield(mg)	R_f	DPPH radical scavenging activity	TBARS formation inhibitory activity	Fe^{2+}ion chelating activity
HM EtOAc fraction (CC H/E/M)					
HM-1 (10% E/H)	721.58		76.24	2.01	41.26
HM-2 (50% E/H)	514.32		80.16	0.61	22.16
HM-3 (70% E/H)	185.6		1.24	4.16	1.26
HM-4 (80% E/H)	1246.35		64.36	0.26	1.07
HM-5 (100% E)	506.98		3.02	0.67	3.16
HM-6 (10% M/E)	899.87		4.11	4.01	0.01
HM-7 (20% M/E)	1523.44		76.11	3.04	11.06
HM-8 (100% M)	2001.55		0.02	10.96	0
HM-1 (PTLC 20% E/H)					
HM-9	4.74	0.25	60.11	0.11	0.06
HM-10	185.69	0.48	12.46	7.94	0.81
HM-11	324.43	0.74	31.06	0.1	1.76
HM-12	201.66	0.9	0.26	9.86	11.01
HM-11 (PTLC 20% E/H)					
HM-13	144.68	0.24	4.46	1.37	0.76
HM-14	6.98	0.56	62.31	NA	NA
HM-15	165.66	0.81	10.01	0.67	0.11
HM-2 (CC H/E/M)					
HM-16 (10% E/H)	15.21		6.27	6.76	0.36
HM-17 (50% E/H)	4.22		70.11	NA	NA
HM-18 (70% E/H)	51.55		4.06	5.34	20.16
HM-19 (80% E/H)	16.44		6.18	NA	NA
HM-20 (100% E)	274.66		70.11	0.57	28.11
HM-21 (10% M/E)	6.88		4.01	NA	NA
HM-22 (20% M/E)	24.22		6.26	3.01	0.51
HM-23 (100% M)	115.87		0.01	4.26	26.26
HM-20 (CC H/E)					
HM-24 (100% H)	2.88		8.49	NA	NA

HM-25 (30% E/H) COMPOUND I	126.78		86.46	0.88	40.25
HM-26 (40% E/H)	16.55		10.1	1.26	4.21
HM-27 (50% E/H)	3.11		9.46	2.36	5.14
HM-28 (100% E)	122.66		10.02	5.16	6.06
HM-4 (CC H/E/M)					
HM-29 (100% H)	64.21		0.01	1.46	7.14
HM-30 (10% E/H)	124.56		0.2	2.16	2.13
HM-31 (20% E/H)	368.99		76.11	0.04	21.46
HM-32 (30% E/H)	16.89		0.46	0.96	0.45
HM-33 (50% E/H)	324.47		26.11	1.27	0.12
HM-34 (100% E)	202.11		48.26	3.16	22.46
HM-35 (5% M/E)	21.01		11.01	4.01	0.1
HM-36 (50% M/E)	112.54		12.21	4.06	4.11
HM-31 (PTLC 20% E/H)					
HM-37	67.44	0.16	48.26	1.56	34.31
HM-38	42.01	0.24	0.16	1.27	0.61
HM-39	25.64	0.36	2.45	5.76	0.72
HM-40	96.32	0.52	6.01	3.17	0.89
HM-41	23.54	0.78	7.24	2.16	1.46
HM-42	102.68	0.96	52.31	1.01	10.26
HM-37 (PTLC 20% E/H)					
HM-43	60.22	0.16	6.66	0.96	10.11
HM-44	4.25	0.92	60.11	NA	NA
HM-42 PTLC 30% E/H)					
HM-45 COMPOUND II	50.54	0.16	49.01	0.76	2.11
HM-46	18.44	0.28	5.24	3.14	3.14
HM-47	31.87	0.54	28.26	8.26	4.06
HM-34 PTLC (10% M/E)					
HM-48	56.14	0.24	62.16	0.11	1.46
HM-49	61.25	0.56	0.12	0.96	0
HM-50 COMPOUND III	81.44	0.86	56.23	1.34	21.06
HM-7 (CC C/M)					
HM-51 (100% C)	600.02		39.4	0.98	0.64
HM-52 (1% M/C)	94.55		38.2	1.26	0.76
HM-53 (10% M/C)	184.11		0.2	1.86	0.51
HM-54 (15% M/C)	434		12.02	6.11	0.46
HM-55 (30 % M/C)	171.01		72.16	0.14	0.81
HM-55 (PTLC 40% E/H)					
HM-56	64.2	0.1	28.24	3.26	1.26
HM-57	10.88	0.5	59.16	0.08	40
HM-58	91.56	0.94	4.01	6.84	0

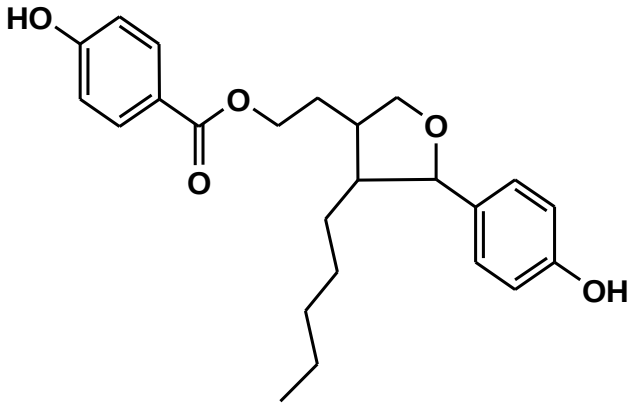
DPPH radical scavenging activity at 0.1 mg/ml is expressed in % (48 h); lipid peroxidation inhibitory activity at 0.1 µg/ml is expressed in MDAEQ/kg sample; Fe^{2+} ion chelating activity at 0.1 mg/ml is expressed in %. NA – NOT ASSAYED ie. the fractions with low yield were evaluated only for DPPH radical scavenging activity. CC – Column chromatography; PTLC – Preparative thin layer chromatography; M – Methanol; E –Ethyl acetate; H – *n*-hexane; C – Chloroform; HM - *Hypnea musciformis*

The results of the chemical investigation of the three pure compounds (HM – 25, HM -45 & HM -50) isolated from the red seaweed, *Hypnea musciformis* are as follows.

8.2.2.1. Structural Characterization of Compound I (HM-25)

Compound I (HM-25)

2-(Tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl)ethyl 4-hydroxybenzoate



Yield	126.8 mg (1.58 %*)
Physical description	White semisolid
Molecular formula	$C_{24}H_{30}O_5$
Molecular weight	398.6984

* % yield based on starting EtOAc extract

The spectroscopic characterization of the pure and active compound (HM-25), 2-(tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate are discussed in detail below.

2-(Tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate: White semisolid; UV (MeOH) λ_{max} (log ε): 247 nm (3.81); TLC (Si gel GF_{254} 15 mm; CC 30% EtOAc:*n*-hexane, v/v); GC (Elite – 5 capillary column 30 m x 0.53 mm i.d.; oven temperature ramp: 60^{o}C for 10 min, rising at 5^{o}C /min to 220^{o}C; 1 ml injection volume/$CHCl_3$) R_t: 16.01 min.; Elemental analysis found: C,72.34;H,7.59;O,20.07; .; IR (KBr, cm^{-1}) ν_{max} 720.29 (C-H ρ), 814.06 (aromatic C-

H δ), 1376.26 (C-H ρ), 1454.38 (C-H δ), 1466.91 (C-H δ), 1650.42 (C=C ν), 1690.21 (C-CO-C ν), 1726.86 (C=O ν), 2955.04 (C-H ν), 3346 (br O-H ν); ^{1}H-NMR (500 MHz, Chloroform-d) δ 7.71(m, 1H), 7.55 – 7.45 (m, 1H), 4.31(t, 2H, J=6.6), 3.95(d, 1H, J=6.3), 1.72(m, 2H), ^{13}C-NMR ($CDCl_3$), ^{1}H-^{1}H-COSY and HMBC data (details under the Table 8.5, Fig. 8.7.1); HRMS (ESI) m/e: 398.6984 calcd. for $C_{24}H_{30}O_5$ 398.1268; found 396.6984 $[M-2H]^{-2}$.

Compound I (HM-25), a new derivative of the substituted heptalenofurandione, was isolated as white semisolid upon repeated column chromatography using silica gel as adsorbent. HM-25 exhibited 86.5 % DPPH radical scavenging activity, 40.2 % Fe^{2+} ion chelating activity and 0.88 MDAEQ/kg TBARS formation inhibitory activity (Table 8.4). The ^{1}H, ^{13}C, DEPT, ^{1}H–^{1}H COSY, HSQC and HMBC NMR spectra of 2-(Tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate is shown in Fig. 8.7.1. Similarly, the ^{1}H, ^{13}C, DEPT, ^{1}H–^{1}H COSY, HSQC and HMBC NMR spectra of 2-(Tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate is shown in Fig. 8.7.1-8.7.8. The ^{1}H NMR in conjugation with ^{13}C-NMR recorded the presence of methylene signals at δ 0.96,1.26, 1.36, 1.44 and 1.72 and the ^{1}H–^{1}H COSY couplings were apparent between these protons assigned to be at H-23/H-22, H-21/20; which support the presence of C-5 skeleton. The relatively downfield shift of the methylene proton at δ 1.72 and the C-20 carbon at δ 27.73 referred to a possible oxygenation in its vicinity. The aromatic protons were assigned to be present at δ 7.4-7.7 and the proton integral of the protons revealed the presence of two aryl rings. The broad IR absorption band (in MeOH) at 3100-3350 cm^{-1} is due to –OH groups in the skeleton exhibit free –OH stretching vibrations, which has been supported by the ^{1}H-NMR signal at about δ 3.15. The exchangeable hydroxyl protons at δ 3.15 (2H, bs in $CDCl_3$) disappeared upon addition of D_2O. The methylene group protons at δ 4.31 and δ 2.33 are assigned to be at C-8 and 9 positions, respectively, and the downfield shift (about δ 0.75) is apparently due to the presence of the possible extended conjugated moiety in its vicinity. A strong HMBC correlation was found between H-8 (δ 4.31)/ C-7 (δ 167) (Table 8.5), which apparently indicates the presence of the carbonyl carbon near the methylene group. The presence of two quaternary carbons at δ 132.96 and δ 162.42 are due to the presence of substituted hydroxybenzyl moiety. The protons of the –CH- groups at δ 2.04, 2.40, and 3.95 are deshielded due

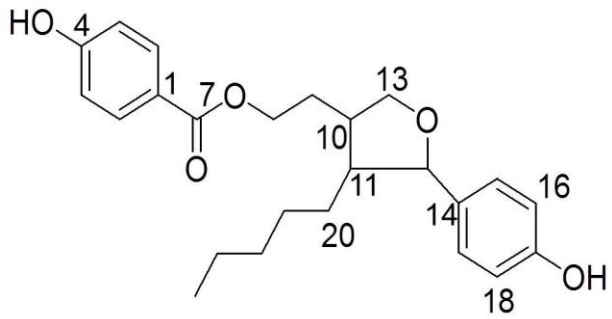
to the possible oxygenation at its vicinity. Also, the $^{1}H–^{1}H$ COSY correlations between H-13 (δ 4.09) /H-10 (δ 2.04)/H-11 (δ 2.40)/ H-12 (δ 3.95), along with the proton and carbon connectivities deduced from HSQC and HMBC experiments (Table 8.5) confirmed the tetrahydrofuran framework. The –CH proton at δ 2.40 (assigned to be at C-11) exhibited strong $^{1}H–^{1}H$ COSY correlations with the methylene protons at δ 1.72, 1.44, 1.36, 1.26 and 0.96 which established the presence of 3-pentyl-tetrahydrofuran moiety in the compound. The carboxyl ester group at the C-7 position of the structure resulted in strong deshielding of the aromatic quaternary –C- at δ 167.68, and therefore, has been assigned to be present at the junction point between the aromatic ring and carboxyl ester group. The methine proton at δ 2.04 is characteristic of the junction point of the tetrahydrofuran ring with that of the side chain ethyl hydroxybenzoate moiety as established by $^{1}H–^{1}H$ COSY correlations and detailed HMBC experiments (Table 8.5). The ^{13}C NMR spectrum of HM-25 in combination with DEPT experiments indicated the occurrence of 24 carbon atoms in the molecule including one ester carbonyl carbon at δ 167.68, three methine carbons between δ 30.57, 29.60 and 72.38 (Table 8.5). The latter was found to be significantly down shielded due to the presence of possible conjugation in the form of the aromatic ring system. The –CH proton at δ 72.38 exhibited HMBC correlation with the aromatic carbon atom assigned to be as C-14 (δ 132.90). It is therefore, the C-C bond exists between the aromatic ring and the substituted furanyl moiety. The low field quaternary signals (^{13}C NMR) is in agreement with that to a quaternary carbon signal carrying the carbonyl groups at C-7 of the structure (δ 167.68) and C-1' of the aromatic carbon (δ 132.96) attached with the ethyl hydroxybenzoate side chain. The aromatic side chain attached to the furanyl group is substituted at C-12 with either a α- or β-oriented proton at the C-12 (δ 3.95) position. The position of the hydroxyl group at C-4 (δ 132.96) was further confirmed from NOESY and HMBC experiments. The point of cyclization of the substituted furanyl ring was indicated by the low-field shift of H-10 at δ 2.04, which has been coupled to the H-9 methylene group at δ 2.33, which also gives clear $^{1}H–^{1}H$ COSY correlation with H-8/H-11/H-13, which support the presence of the 3-butyl-tetrahydrofuran moiety in the compound. The proton and carbon connectivity deduced from HSQC and HMBC experiments confirmed the 3-pentyl-tetrahydrofuran framework attached to the aromatic side chain at the 12^{th} position of the compound. In the HMBC spectrum, it

was observed that H-13 (δ 4.09)/C-8 (δ 65.55), C-12 (-CH, d, δ 72.38); H-15 (δ 7.71)/C-12 (δ 72.38) were correlated with each other (Table 8.5), which support the presence of the 4-(3-pentyl-tetrahydrofuran-2-yl) phenol moiety. The $–CH_2$ proton at C-8 (δ 4.31) appeared to demonstrate long range HMBC correlation (Fig. 8.7.1) with ester carbonyl carbon at δ 167.68 (C-7). The HMBC spectrum of HM-25 also revealed connections of the protons at C-8 (δ 4.31) to the methylene carbon at δ 65.55. This along with extensive 2D NMR analyses assigned the presence of ethyl hydroxybenzoate moiety in the compound. The relative stereochemistry of the chiral centres particularly that of C-10, 11 and 12 of the furanyl ring framework was deduced from the NOESY spectrum of the compound and the J-values. NOE couplings were observed between Hα-12 (δ 3.95)/Hα-10 (δ 2.04) thus indicating that these groups must be equatorial and on the α-side of the molecule. The methine proton at C-11 group did not exhibit NOE interactions with H-12 and H-10, which is at the α-face of the molecule, thereby indicating that H-11 is at the axial disposition. An interaction through space of the hydroxyl protons at C-1 (δ 3.25, 1H, bs) and C-17 (δ 3.45, 1H, bs) with H-11 (δ 2.40) and Hβ-8 (δ 4.31) is only possible for the β-orientation of the hydroxyl groups under observation.

The IR absorption band (in MeOH) exhibited free –OH stretching vibrations near 3346.0 cm^{-1}. The bending vibration bands near 1726.86 cm^{-1} denotes the ester carbonyl absorption. The olefinic (C=C), and ether (C-O-C) groups have been symbolized by the absorption bands at 1650.42 and 1690.21 cm^{-1}. The IR spectrum revealed broad absorption band at ν_{max} 3400 to 3100 cm^{-1}, attributed to hydroxyl functionality. The ultraviolet absorbance at λ max (log e) 247 nm (3.81) was assigned to a chromophore with extended conjugation. Its mass spectrum exhibited a molecular ion peak at m/e 398 (HRESIMS m/e 398.6984 $[M-2H]^{-2}$; D 0.0 amu), which in combination with its ^{1}H and ^{13}C NMR data (Table 8.5) indicated the elemental composition of $C_{24}H_{32}O_5$ as 2-(4-pentyl-tetrahydro-5-(4-hydroxyphenyl) furan-3-yl) ethyl 4-hydroxybenzoate with ten degrees of unsaturation. One degree of unsaturation from the carbonyl group (δ 167.68), six degrees of unsaturation from the double bonds of the two aromatic ring system (δ 128-162.42), two degrees of unsaturation from two aromatic rings, and the balance one degree from one ring system. The molecular ion peak at m/e 398.49 appeared to undergo elimination of 4-(tetrahydrofuran-2-yl) phenol ($C_{10}H_{12}O_2^{\bullet+}$, m/e: 164.2) to yield 3, 4-dihydro-6-

hydroxyisochromen-1-one at m/e 164.16 ($C_9H_8O_3^{\bullet+}$), which undergoes rearrangement to afford a fragment with m/e 91.13 (tropylium ion, C_7H_7+). Appearance of the fragment at m/e 68.07 indicates the presence of furanyl moiety ($C_4H_4O^{\bullet+}$), resulted from the intramolecular rearrangement of 4-(tetrahydrofuran-2-yl) phenol ($C_{10}H_{12}O_2^{\bullet+}$, m/e: 164.2).

Table 8.5 NMR spectroscopic data of 2-(tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate in $CDCl_3$.[a]



Carbon no.	^{13}C NMR (DEPT)	H	δ^{1}H NMR (int., mult., J in Hz)[b]	^{1}H-^{1}H COSY	HMBC (^{1}H-^{13}C)
1	132.96				
2	128.83	2H	7.71(m,1H)	H-3	
3	128.70	3H	7.55-7.45(m,1H)		C-7
4	162.42				
5	130.90	5H	7.55 – 7.45 (m,1H)	H-6	C-7
6	130.83	6H	7.71(m,1H)		
7	167				
8	65.55	8H	4.31(t,2H,J=6.6)	H-9	C-7,C-10,C-11
9	38.05	9H	2.33(m, 2H)	H-8,10	C-10
10	30.57	10H	2.04(m,1H)	H-11,H-13	
11	29.60	11H	2.40(m,1H)	H-20,H-12,H-10	
12	72.38	12H	3.95(d,1H,J=6.3)		C-21
13	71.76	13H	4.09 (d,2H,J=6.7)		C-12
14	132.90				
15	130.75	15H	7.71(m,1H)	H-16	C-12
16	130.67	16H	7.55 – 7.45 (m,1H)	H-19	
17	132.31		(4.02,OH)		
18	128.70	18H	7.55 – 7.45 (m, 1H)	H-19	
19	128.69	19H	7.71(m,1H)		C-18
20	27.73	20H	1.72(m,2H)	H-11,H-21	C-11
21	22.69	21H	1.44(m,2H)	H-22	
22	19.18	22H	1.36(m,2H)	H-23	
23	14.13	23H	1.26(m,2H)	H-24	C-21,C-22
24	13.73	24H	0.96(m,3H)		C-23

[a] NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers. [b] Values in ppm, multiplicity and coupling constants (J¼ Hz) are indicated in parentheses. Assignments were made with the aid of the ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY experiments.

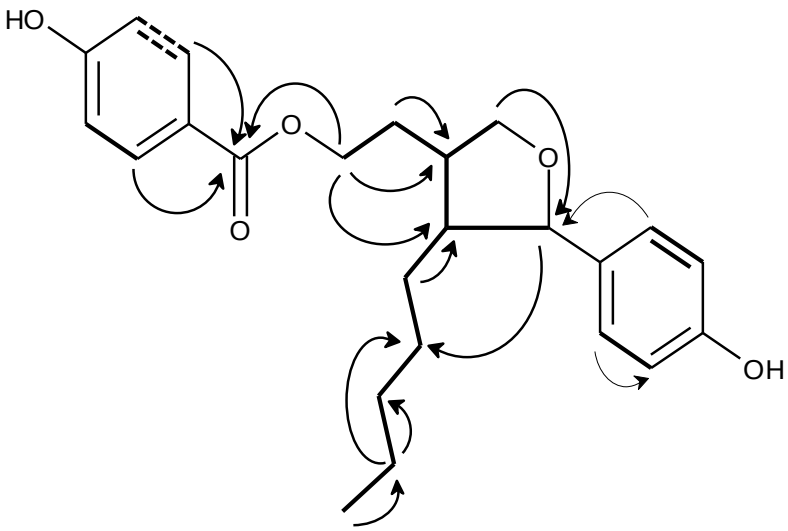


Fig. 8.7.1. ^{1}H–^{1}H COSY and HMBC correlations of 2-(tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate. The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

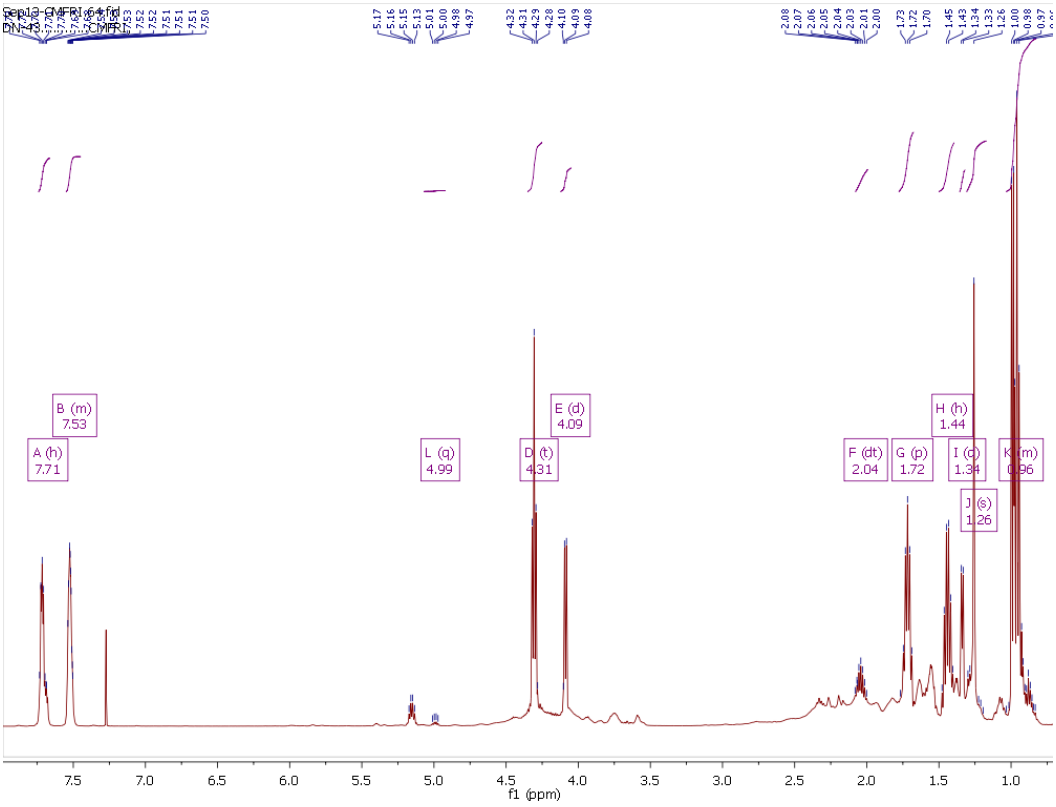


Fig. 8.7.2. Proton NMR spectrum of 2-(tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate

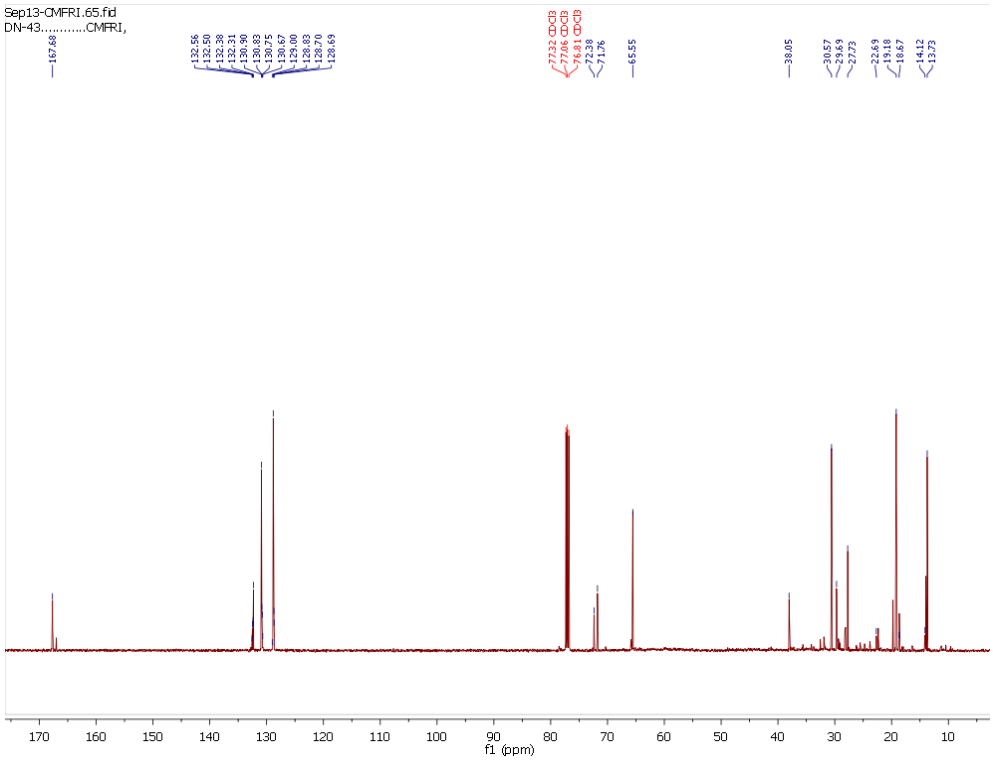


Fig. 8.7.3. ^{13}C NMR spectrum of 2-(tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate

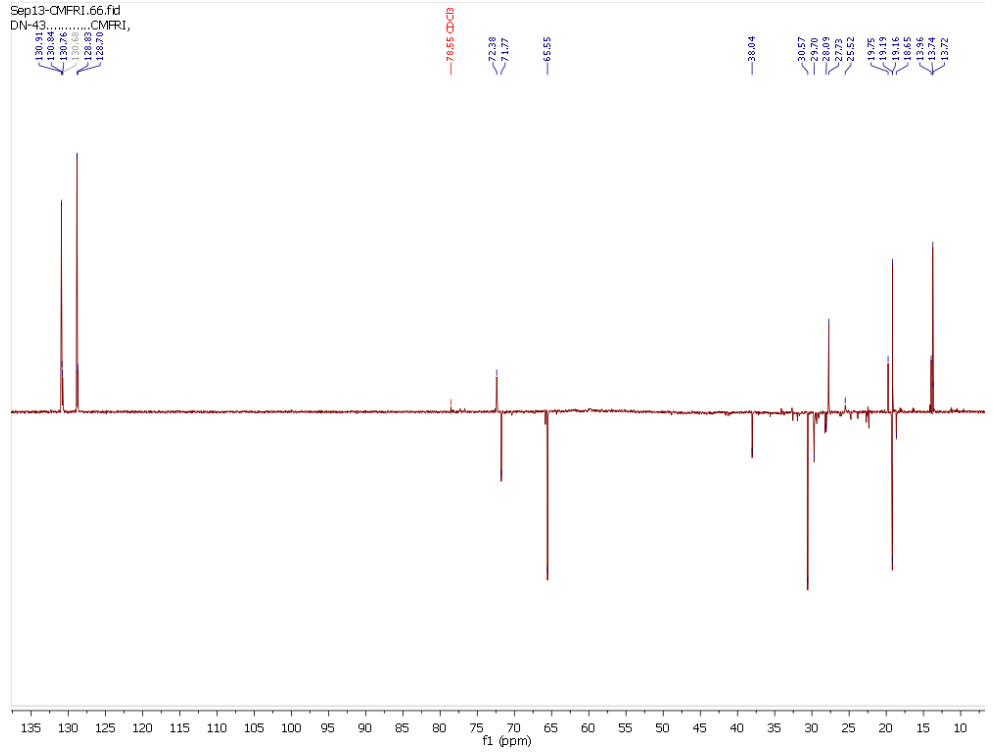


Fig. 8.7.4. DEPT spectrum of 2-(tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate

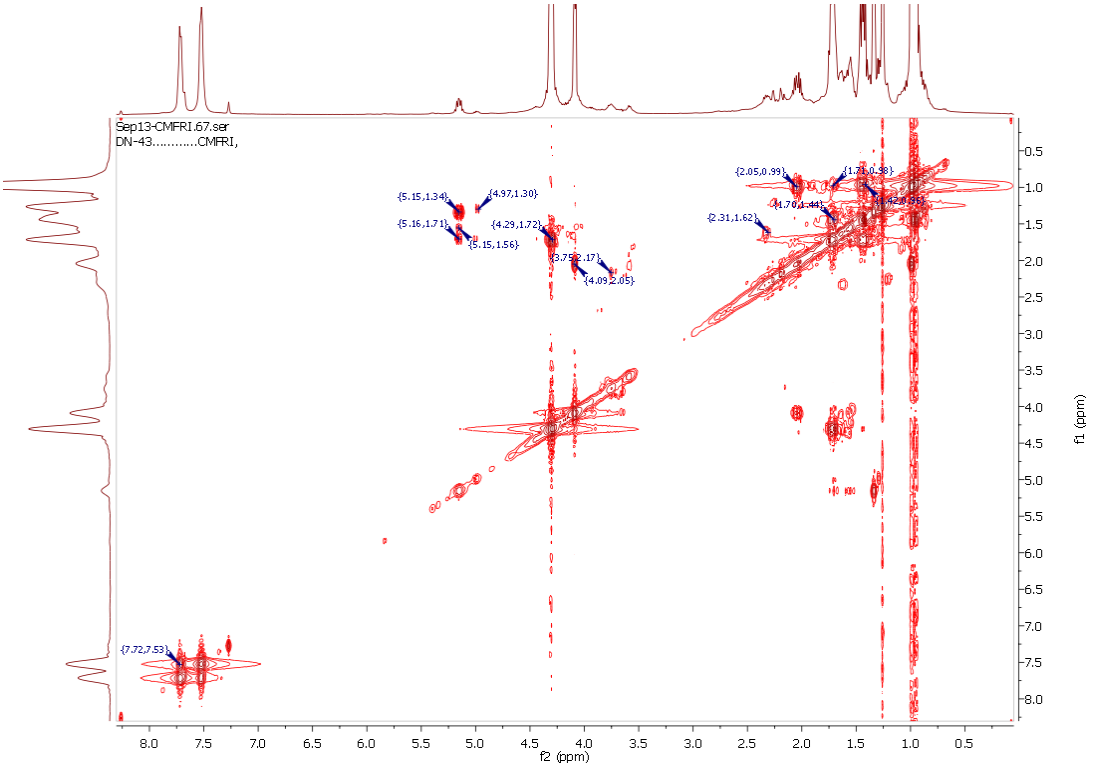


Fig. 8.7.5. ^{1}H–^{1}H COSY spectrum of 2-(tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate

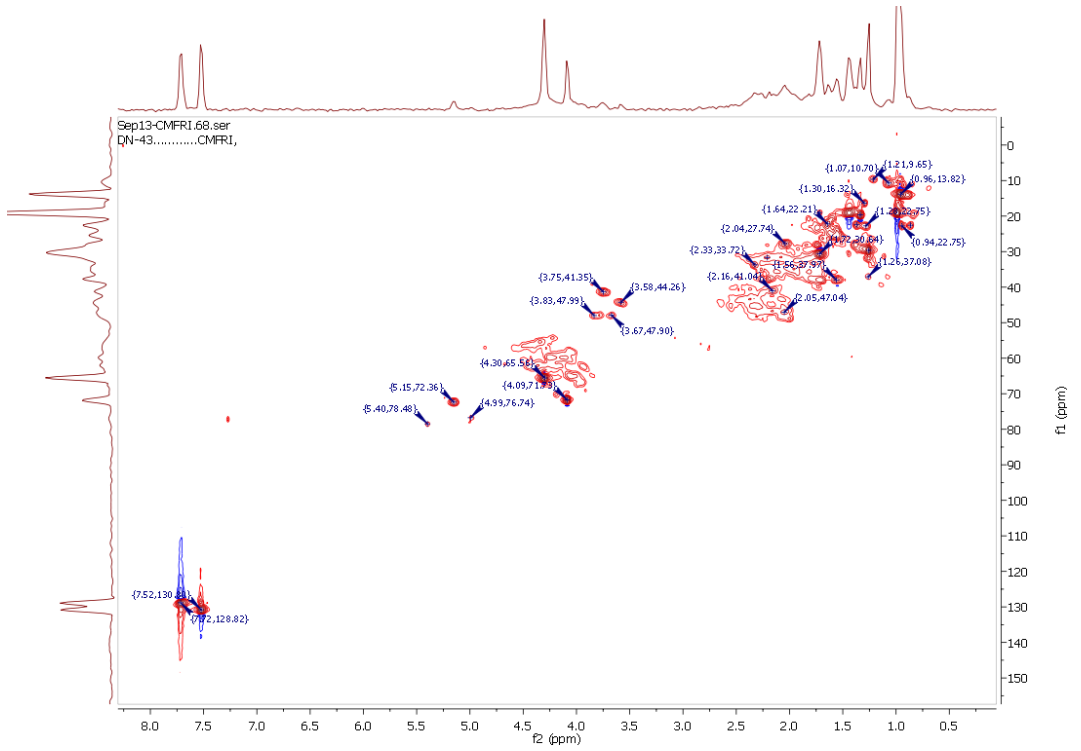


Fig. 8.7.6. HSQC spectrum of 2-(tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate

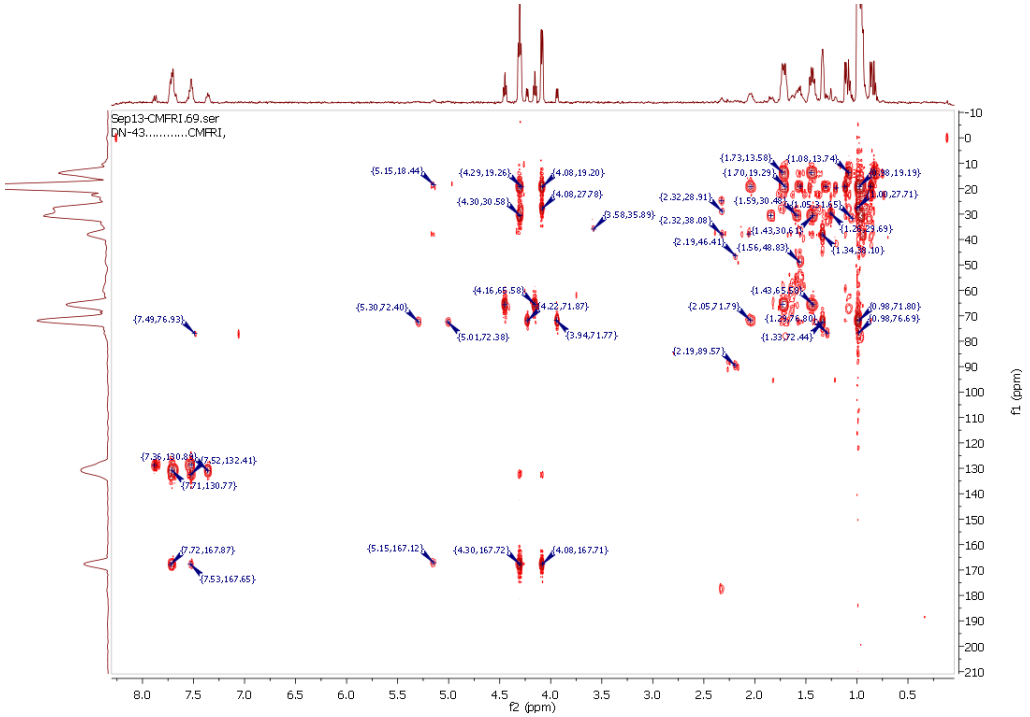


Fig. 8.7.7. HMBC spectrum of 2-(tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate

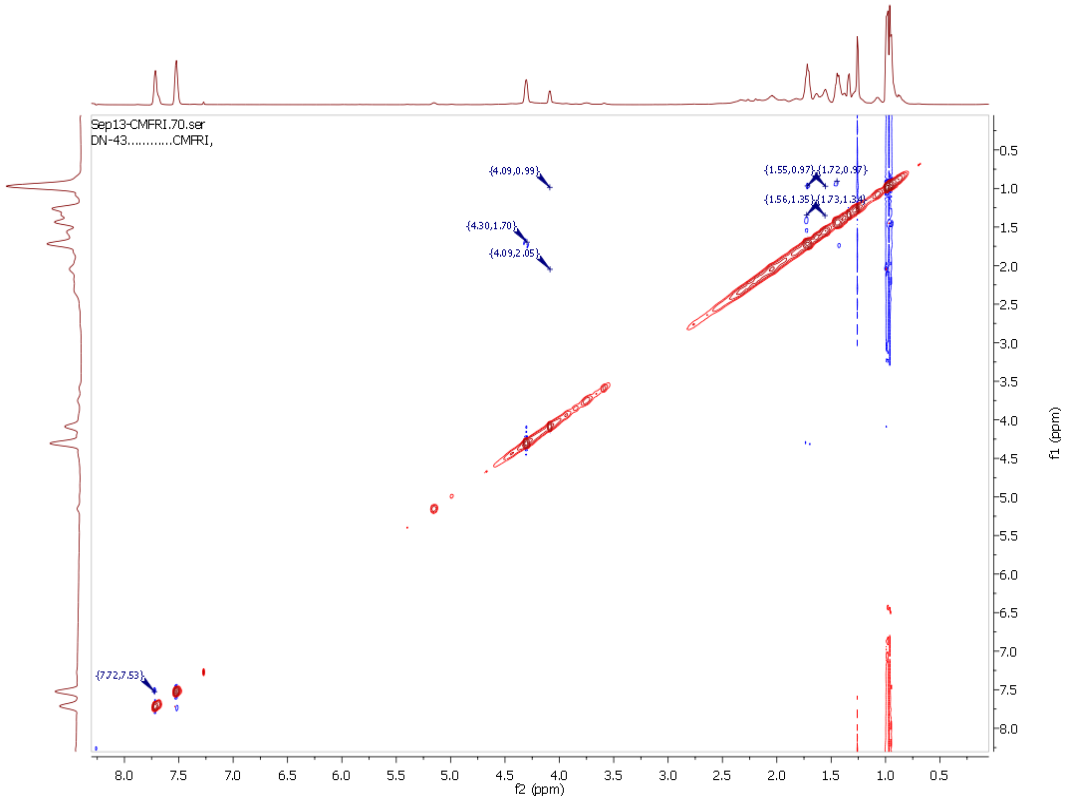


Fig. 8.7.8. NOE spectrum of 2-(tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate

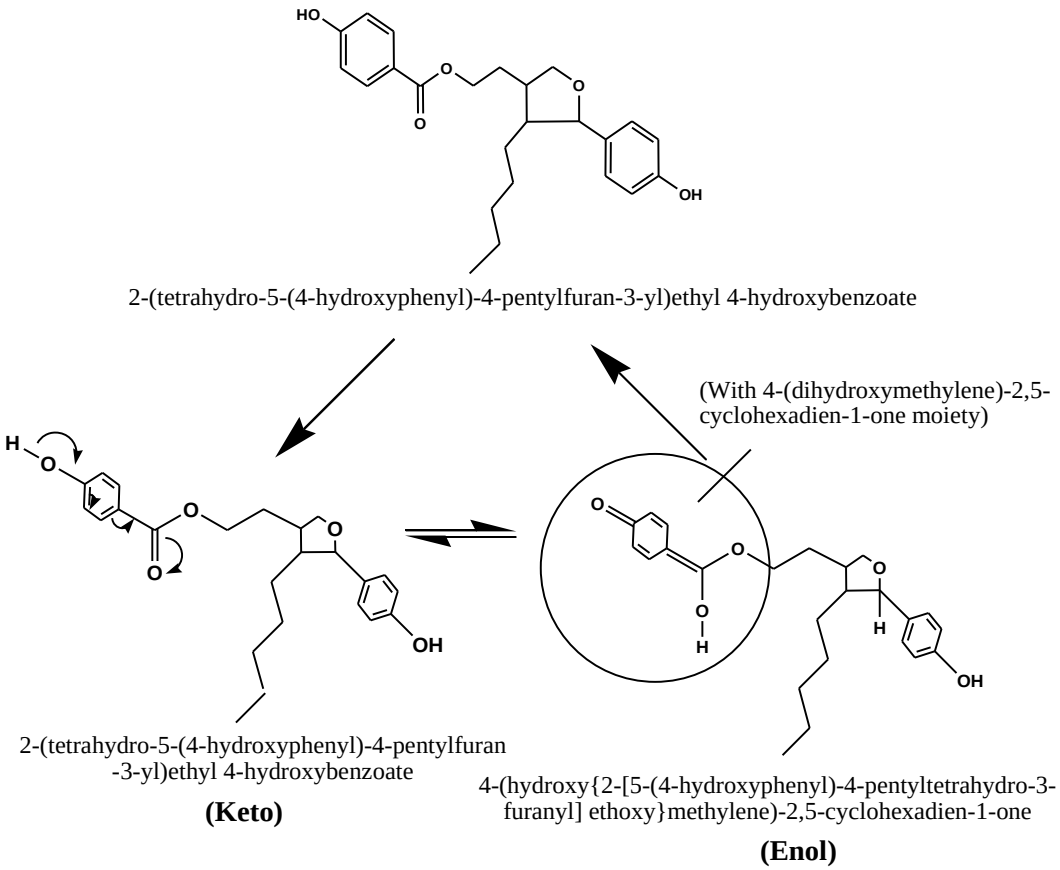


Fig. 8.7.9. Keto-enol tautomerism of of 2-(tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate

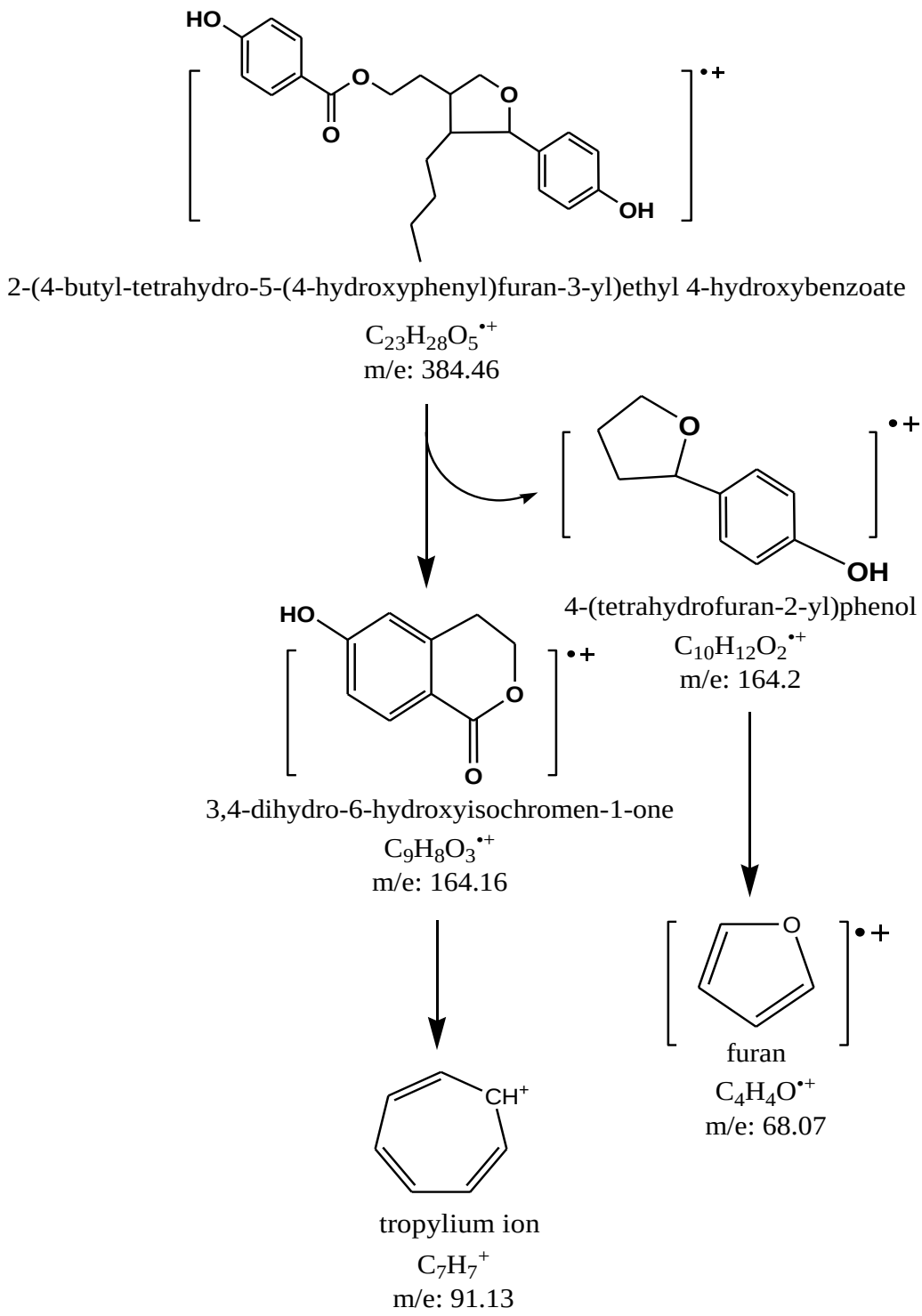


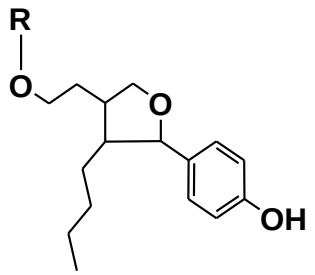
Fig. 8.7.10 Mass fragmentation pattern of 2-(tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate

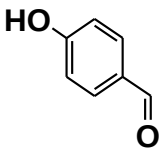
Investigations into the natural products of marine invertebrates have yielded a large number of furano derivatives, some of which have shown fascinating biological activities. These furano compounds were reported to be linked through certain oxidative transformations (Roethle & Trauner 2008). Among the furan substituted marine natural products, the furanocembranoids featuring a canonical cembrane skeleton and a 14-membered carbocyclic ring as well as a furan heterocycle encompassing C3–C6 were found to be predominant. Furan and lactone moieties are commonly found in the compounds isolated from marine natural products. The vast majority of furanocembranoids have been isolated from gorgonian corals. The furan containing marine natural products were reported to possess potent biological activity such as inhibitor of the nicotinic acetylcholine receptor (Roethle & Trauner 2008). 7-Methoxy-2, 3-dimethylbenzofuran-5-ol isolated from the fungus *Malbranchea cinnamomea* HKI 0286 was found to possess potential antioxidative properties and inhibitor of peroxidase as key enzyme of oxygen radical generation (Schlegel *et al.,* 2003). The antiviral activity of furan-2-yl acetate extracted from *Streptomyces* spp. was studied in cultured Sahul Indian Grouper Eye (SIGE) cells infected with fish nodavirus (FNV) (Suthindhiran *et al.,* 2011). A fatty acid containing a furan ring was detected in the cellular lipids of marine bacteria, *Shewanella putrefaciens*, *Marinomonas comunis*, *Enterobacter agglomerans*, and *Pseudomonas fluorescens* (Shirasaka *et al.,* 1995). Cytotoxic furanosesterterpenes were isolated from a marine sponge *Psammocinia* sp. by bioactivity-guided fractionation. These compounds displayed significant inhibition of DNA replication and moderate antioxidant profile, and were showed toxicity to human tumor cell line SK-MEL-2 (Choi *et al.,* 2004).

The activity of 2-(tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate appeared to be due to the tautomeric conversion of 2-(tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate (with 4-hydroxybenzoate group) and 4-(hydroxy{2-[5-(4-hydroxyphenyl)-4-pentyltetrahydro-3-furanyl] ethoxy}methylene)-2,5-cyclohexadien-1-one (with 4-(dihydroxymethylene)cyclohexa-2,5-dienone group) in the compound resulting in highly delocalized electrons that are potentially function as antioxidative centres of the molecule. Other compounds from *Hypnea musciformis* with no 4-hydroxybenzoate moiety (4-(3-butyl-tetrahydro-4-(2-methoxyethyl)furan-2-

yl)phenol and 4-(4-(2-(3-methylenehex-5-enyloxy)ethyl)-3-butyl-tetrahydrofuran-2-yl)phenol) did not show equivalent antioxidative activity proving that the later has major role to scavenge the free radicals species responsible for oxidation activity.

The compound with hexen-3-one group substituting the 4-hydroxybenzoate moiety was found to possess one each of keto and olefinic groups. However, these groups are not in conjugation, and therefore, don't experience and electron delocalization in the side chain of the compound (4-(4-(2-(3-methylenehex-5-enyloxy) ethyl)-3-butyl-tetrahydrofuran-2-yl) phenol). This apparently results in comparatively lower antioxidative activity of 4-(4-(2-(3-methylenehex-5-enyloxy) ethyl)-3-butyl-tetrahydrofuran-2-yl) phenol than 2-[4-butyl-5-(4-hydroxyphenyl) tetrahydro-3-furanyl] ethyl 4-hydroxybenzoate.

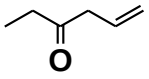


R = 

2-[4-butyl-5-(4-hydroxyphenyl) tetrahydro-3-furanyl] ethyl 4-hydroxybenzoate

= $-CH_3$

4-(3-Butyl-tetrahydro-4-(2-methoxyethyl)furan-2-yl)phenol

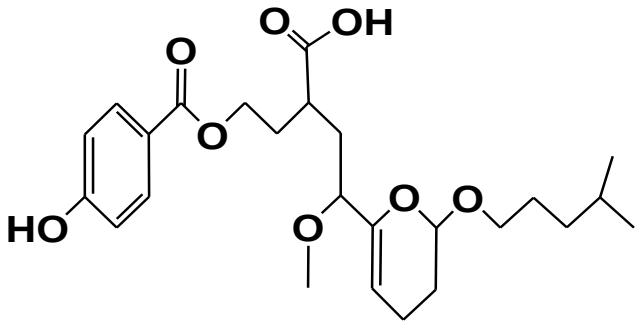
= 

4-(4-(2-(3-methylenehex-5-enyloxy)ethyl)-3-butyl-tetrahydrofuran-2-yl)phenol

8.2.2.2. Structural Characterization of Compound II (HM-45)

Compound II (HM-45)

2-2-[(4-Hydroxybenzoyl) oxy] ethyl-4-methoxy-4-2-[(4-methylpentyl)oxy]-3,4-dihydro-2H-6-pyranylbutanoic acid



Yield	50.5 mg (0.63 %*)
Physical description	Yellow semi-solid
Molecular formula	$C_{25}H_{36}O_8$
Molecular weight	464.4268

* % yield based on EtOAc extract

The spectroscopic characterization of the pure and active compound (HM-45), 2-2-[(4-hydroxybenzoyl) oxy] ethyl-4-methoxy-4-2-[(4-methylpentyl) oxy]-3, 4-dihydro-2H-6-pyranylbutanoic acid are discussed in detail below.

2-2-[(4-hydroxybenzoyl) oxy] ethyl-4-methoxy-4-2-[(4-methylpentyl)oxy]-3,4-dihydro-2*H*-6-pyranylbutanoic acid: Yellow semisolid; UV (MeOH) λ_{max} (log ε): 242 nm (4.12); TLC (Si gel GF_{254} 15 mm; 20% EtOAc/*n*-hexane, v/v) R_f: 0.16; R_t:14.45 min.; GC (Elite – 5 capillary column 30 m x 0.53 mm i.d.; oven temperature ramp: 60^{o}C for 10 min, rising at 5^{o}C /min to 220^{o}C; 1 ml injection volume/$CHCl_3$) R_t: 14.45 min.; Elemental analysis found: C,64.44;H,7.81;O,27.55; IR (KBr, cm^{-1}) ν_{max} 812.06 (aromatic C-H δ), 1310.25 (C-O ν), 1376.26 (C-H ρ), 1661.91 (C=C ν), 1690.21 (C-CO-C ν), 1740.96 (C=O(O) ν), 2923.22 (C-H ν), 3010.12 (aromatic C-H ν), 3208 (O-H ν); ^{1}H-NMR (500 MHz, Chloroform-d) δ 9.76 (t, J = 1.9 Hz, 1H), 9.36 (s, OH), 7.82 – 7.66 (m, 1H), 7.53 (tdd, J = 5.7 Hz, 1H), 5.15(t, 1H, J=6.7 Hz), 4.31 (t, J = 6.7 Hz, 2H), 4.22 (dd, J = 8.8 Hz, 1H), 3.65 (s, 3H), 2.83 (m, 2H), 1.72(m, 2H), 0.94(m, 3H), 0.84(m, 3H) (see details in Table 8.6); ^{13}C-NMR ,^{1}H-^{1}H-

COSY and HMBC data (details under the Table 8.6, Fig. 8.8.1); HRMS (ESI) *m/e*: 465.5689 calcd. for $C_{25}H_{37}O_8$ 465.4268; found 465.5689 $[M+H]^+$ (Fig. 8.8.9).

A new derivative of the pyranylbutanoic acid was isolated as yellowish liquid upon chromatography over silica columns. As evident from Table 8.4, compound 2 exhibited 49.0 % DPPH radical scavenging activity, 2.1 % Fe^{2+} ion chelating activity and 0.76 MDAEQ/kg TBARS formation inhibitory activity (Table 8.4). The ^{1}H-NMR in conjugation with ^{13}C-NMR recorded the presence of methyl signals at δ 0.8-0.9 and δ 3.65. The former is assigned to be due to the terminal methyl groups, whereas the methyl group at δ 3.65 shifted downfield due to the electronegative group (oxygen). The presence of the methoxy group is confirmed by the singlet. The DEPT spectrum confirmed the presence of methine group at δ 19.19 bearing the proton at δ 1.10. In the ^{1}H–^{1}H COSY spectrum, couplings were apparent between the protons at δ 0.94 (H-24)/ δ 0.84 (H-23)/ δ 1.10 (H-22)/, δ 1.32 (H-21)/ δ1.96 (H-20, which support the presence of 2-methylpentane moiety. The methylene signal at δ 4.09 (H-19) appeared downfield due to the presence of multiple electronegative systems at close proximity. Three methylene groups have been assigned to occupy at the C8-9 positions, and the one with δ 4.31 shifted downfield due to the presence of an extended conjugation probably linked to an aromatic moiety. The HMBC correlation of the proton at δ 4.31 with the carbon atom at δ 167.72 apparently indicates the presence of -C=O(O). HSQC and HMBC experiments revealed that the ester group linked to the methylene group at δ 4.31 is attached to the substituted aryl ring. The aromatic protons showed their characteristic signals at δ 7.4-7.8 expect that with significant downfield shift at δ 9.76. This evidently indicates the presence of –OH substitution in the benzene ring. Extensive HMBC and HSQC experiments revealed the presence of hydroxybenzoate moiety in the compound. ^{1}H–^{1}H COSY experiments revealed that the protons at δ 4.31 correlate with the methylene protons at δ 1.72 (assigned to be as H-9) and that at δ 2.32 (H-10), the latter is assigned to be attached to a strongly electronegative group. HMBC correlations were apparent

between H-9 (δ 1.72) with that of a carboxyl carbon at δ 179.99, which apparently indicates the presence of acetoxybutanoic acid moiety. The –CH- proton appeared at δ 4.22 is due to the presence of the methoxy group and have been assigned to be present at the C-25 position of the structure. The carboxyl group at the C-11 position of the compound resulted in strong deshielding of the –CH- proton at δ 2.32 (H-10), and therefore, has been assigned to be present at the C-10 position of the structure. The chemical shift of the protons at δ 1.53, 2.83, 5.37 and 5.15 along with detailed 2D NMR experiments established the presence of 3,4-dihydro-2H-pyran moiety. The methine proton H-18 (gives triplet) at δ 5.15 is characteristic of the junction point of the 3,4-dihydro-2H-pyran moiety with that of the side chain O- methylpentan group. The ^{1}H-NMRspectrum showed two exchangeable hydroxyl proton at δ 3.20 (bs in $CDCl_3$) and δ 3.35 (1H, bs in $CDCl_3$), which disappeared upon addition of D_2O. The ^{13}C-NMR spectrum of the HM-45 in combination with DEPT experiments indicated the occurrence of 25 carbon atoms in the molecule including two carbonyl carbon at δ 179.99 (C-11) and δ 167.72 (C-7), and olefinic carbons at δ 128.11 (C-15) and 138.77 (C-14; Table 8.6). The ^{13}C NMR spectrum of the purified compound displayed two quaternary carbon atoms (δ 167.72, 179.99) bearing the carbonyl groups, along with that one bearing the –OH group (δ 132.32). The low field quaternary signals (^{13}C NMR) is in agreement with that to a quaternary carbon signal carrying the carbonyl groups at C-7 of the structure and this was supported by the relatively downfield shift of the H-8 signal (δ 4.31), which referred to a possible oxygenation in its vicinity. The position of the hydroxyl group at C-4 was further confirmed from the HSQC, NOESY, and HMBC spectra. The proton and carbon connectivity deduced from HSQC and HMBC experiments confirmed the O-methylpentan framework attached to the 3, 4-dihydro-2H-pyran moiety at the 18th position of the ring structure. The H–H and C–H connectivities apparent in the ^{1}H–^{1}H COSY and HMBC spectra respectively indicate that one of the eight unsaturations was due to the 3,4-dihydro-2H-pyran ring framework. In the HMBC

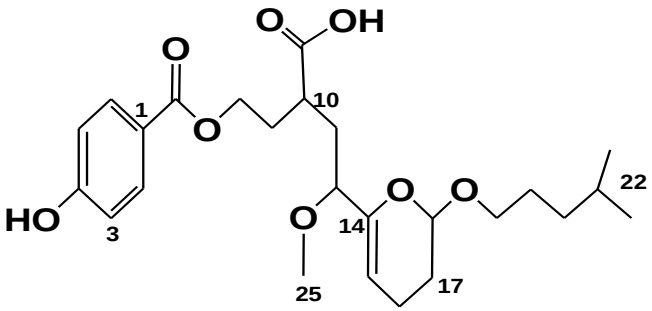
spectrum, it was observed that H-17 (δ 1.53)/C-15 (δ 128.11); H15 (δ 5.37)/C-14 (δ 138.77), 16 (δ 29.70), 17 (δ 22.70); H-17 (δ 1.53)/C-16 (δ 29.70) were correlated with each other (Table 8.6), which support the presence of 3, 4-dihydro-2H-pyran moiety. The detailed HMBC spectral analyses demonstrating the presence of 2-2-[(4-hydroxybenzoyl) oxy] ethyl-4-methoxy-4-2-[(4-methylpentyl) oxy]-3, 4-dihydro-2*H*-6-pyranylbutanoic acid has been represented under the Table 8.6. The geometric isomerisms of the olefinic protons have been established by the 1H coupling constants suitable for the geometries (Z form). The relative stereochemistry of the chiral centre, particularly that of C-4 carrying the hydroxyl group of the framework and that of C-10, was deduced from the NOESY spectrum of the compound and the J-values. NOE couplings were observed between Hα-18/Hα-10 thus indicating that these groups must be equatorial and on the α-side of the molecule. Therefore, the C-1 carboxyl group is axial and β-oriented. NOE correlations between Hβ-13/Hβ-9 indicated the close proximity of these groups and their β-disposition. The methine proton at C-13 group did not exhibit NOE interactions with H-18 and H-10, which are at the α-face of the molecule, thereby indicating that H-13 is at the axial disposition. An interaction through space of the hydroxyl protons at C-4 and C-11 is only possible for the α-orientation of the quaternary hydroxyl group on C-4 and C-11.

The IR absorption band (in MeOH) at 3455 cm^{-1} is due to –CH stretching vibrations. The bending vibration bands near 1740.96 cm^{-1} denotes the ester carbonyl absorption. The olefinic (C=C) group has been symbolized by the absorption bans at 1661.91 cm^{-1}. The IR spectrum revealed broad absorption band at ν_{max} 3500 to 3000 cm^{-1}, attributed to hydroxyl functionality, and to olefinic system (1661.91cm^{-1}). The carboxyl functionality has been denoted by the absorption signal at 1740.96cm^{-1}. The ultraviolet absorbance at λ max (log e) 242 nm (4.12) was assigned to a chromophore with extended conjugation. Its mass spectrum exhibited a molecular ion peak at m/e 464 (HRESIMS m/e 464.5689$[M]^{+}$;D 0.0amu) which in combination with its ^{1}H and

^{13}C NMR data (Table 8.6) indicated the elemental composition of $C_{25}H_{36}O_8$ as 2-2-[(4-hydroxybenzoyl) oxy] ethyl-4-methoxy-4-2-[(4-methylpentyl) oxy]-3, 4-dihydro-2*H*-6-pyranylbutanoic acid with eight degrees of unsaturation. Three degree of unsaturation from the substituted aryl ring (δ 128-162), and the two degree of unsaturation each from the carbonyl (δ 167.72) and carboxyl groups (δ 179.99, ^{1}H-NMR COOH δ 9.36). One degree of unsaturation has been assigned to be due to the presence of the olefinic bond at δ 128.11. The remaining two degrees of unsaturation were assigned to be due to the presence of two rings, one of which is aromatic ring.

The molecular ion peak at m/e 464.55 appeared to undergo elimination of 4-methylpentan-1-ol ($C_6H_{14}O$•+, m/e: 102.17) to yield 5-(5, 6-dihydro-4H-pyran-2-yl) pentyl benzoate at m/e 274.35 ($C_{17}H_{22}O_3^{\bullet+}$), which undergoes elimination of methyl benzoate ($C_8H_6O_2^{\bullet+}$, m/e: 134.13) to afford a fragment with m/e 84.12 ($C_5H_8O^{\bullet+}$). Appearance of the fragment at m/e 154.25 indicates the presence of 3, 4-dihydro-6-pentyl-2H-pyran moiety ($C_{10}H_{18}O^{\bullet+}$), resulted from the intramolecular rearrangement of 5-(5, 6-dihydro-4H-pyran-2-yl) pentyl benzoate ($C_{17}H_{22}O_3^{\bullet+}$, m/e: 274.35).

Table 8.6 NMR spectroscopic data of 2-2-[(4-hydroxybenzoyl) oxy] ethyl-4-methoxy-4-2-[(4-methylpentyl) oxy]-3, 4-dihydro-2*H*-6-pyranylbutanoic acid in $CDCl_3$.[a]



Carbon no.	^{13}C NMR (DEPT)	H	δ^{1}H NMR (int., mult., J in Hz)[b]	^{1}H-^{1}H COSY	HMBC (^{1}H-^{13}C)
1	132.51				
2	130.91	2H	7.82 – 7.66 (m, 1H),	H-3	C-7,3
3	128.84	3H	7.53 (tdd, 1H, J=5.7,3.4,1.9)		C-4
4	132.32		9.76 (t, J = 1.9, OH)		
5	128.71	5H	7.53 (tdd,1H,J = 5.7, 3.4,1.9)	H-6	
6	130.75	6H	7.82 – 7.66 (m,1H)		
7	167.72				
8	65.57	8H	4.31 (t,2H,J=6.7H)	H-9	C-7,9,10

9	30.58	9H	1.72(m,2H)	H-10	C-10
10	38.05	10H	2.32(m,1H)	H-11,H-12	C-11,12
11	179.99		9.36(s,OH)		
12	27.73	12H	1.62(m,2H)	H-13	
13	59.61	13H	4.22(dd,1H)		C-14,9
14	138.77				
15	128.11	15H	5.37(m,1H)	H-16	C-16
16	29.70	16H	2.83(m,2H)	H-17	
17	22.70	17H	1.53(m,2H)	H-18	C-16
18	72.41	18H	5.15(t,1H,J=6.7)		
19	71.79	19H	4.09(m,2H)	H-20	C-21
20	31.93	20H	1.96(m,2H)	H-21	C-19
21	19.76	21H	1.32(m,2H)	H-22	
22	19.19	22H	1.10(m,1H)	H-23,H-24	C-21
23	14.12	23H	0.84(m,3H)		C-21
24	13.73	24H	0.94(m,3H)		C-22
25	43.76	25H	3.65(s,3H)		C-13

[a] NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers.

[b] Values in ppm, multiplicity and coupling constants (J¼ Hz) are indicated in parentheses. Assignments were made with the aid of the ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY experiments.

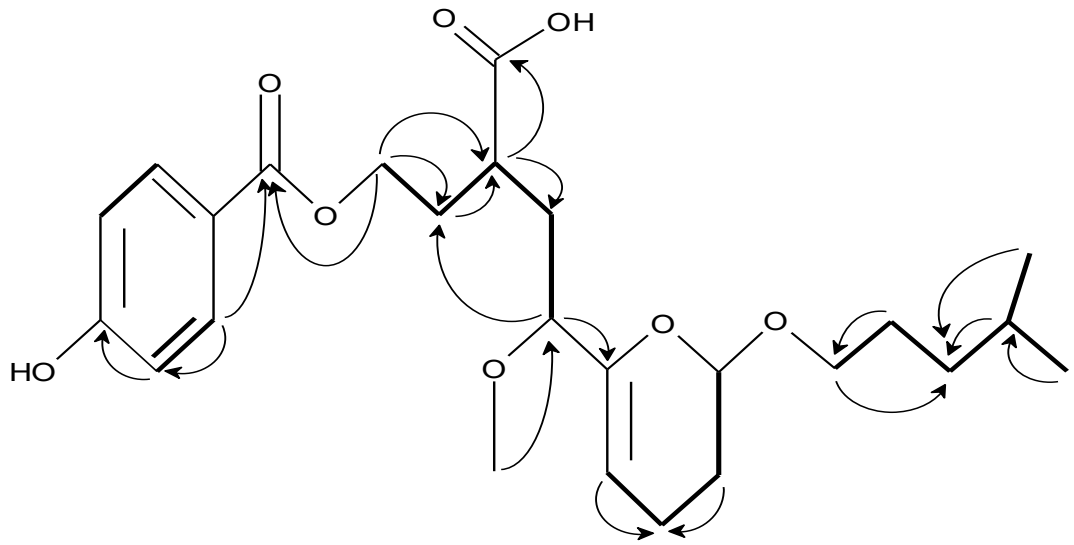


Fig. 8.8.1 ^{1}H—^{1}H COSY and HMBC correlations of 2-2-[(4-hydroxybenzoyl) oxy] ethyl-4-methoxy-4-2-[(4-methylpentyl) oxy]-3, 4-dihydro-2H-6-pyranylbutanoic acid. The key 1H-1H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

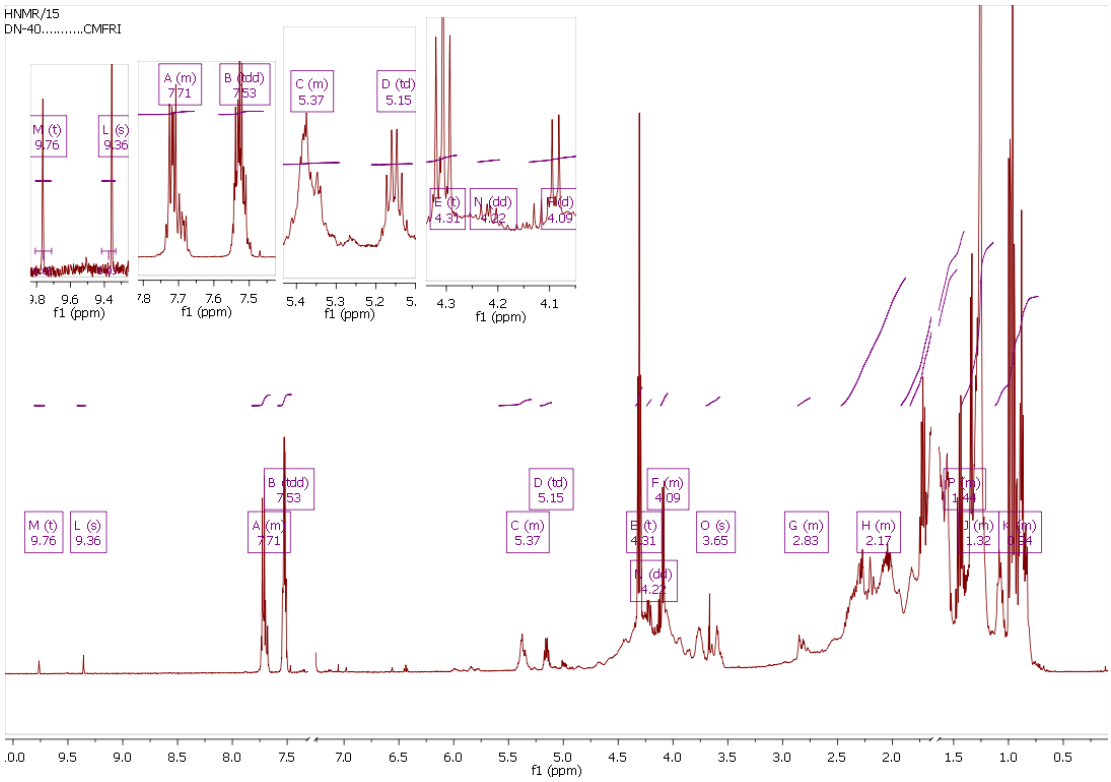


Fig. 8.8.2. Proton NMR spectrum of 2-2-[(4-hydroxybenzoyl) oxy] ethyl-4-methoxy-4-2-[(4-methylpentyl) oxy]-3, 4-dihydro-2H-6-pyranylbutanoic acid

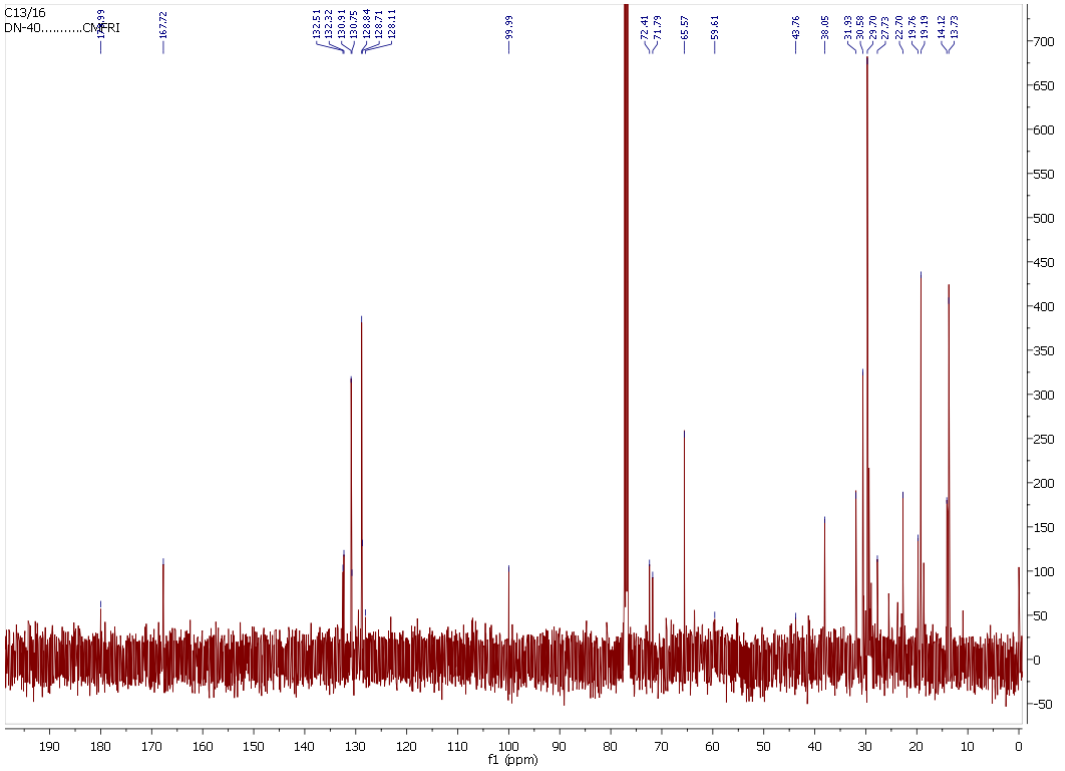


Fig. 8.8.3. ^{13}C NMR spectrum of 2-2-[(4-hydroxybenzoyl) oxy] ethyl-4-methoxy-4-2-[(4-methylpentyl) oxy]-3, 4-dihydro-2H-6-pyranylbutanoic acid

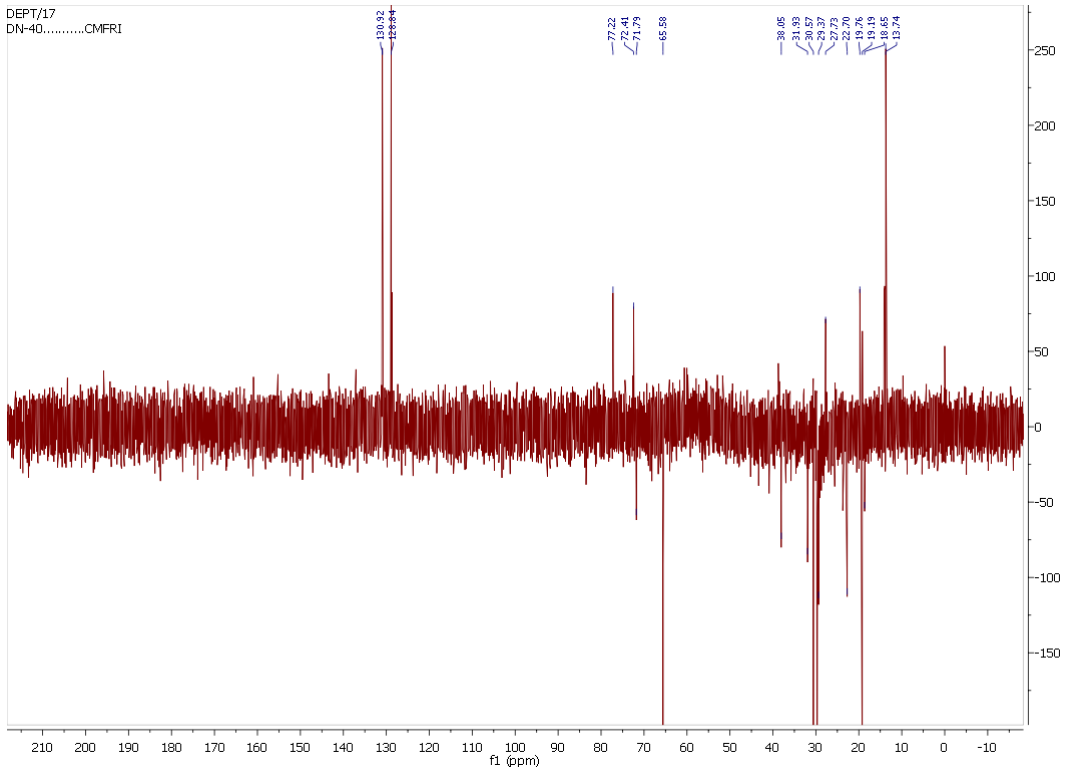


Fig. 8.8.4. DEPT spectrum of 2-2-[(4-hydroxybenzoyl) oxy] ethyl-4-methoxy-4-2-[(4-methylpentyl) oxy]-3, 4-dihydro-2H-6-pyranylbutanoic acid

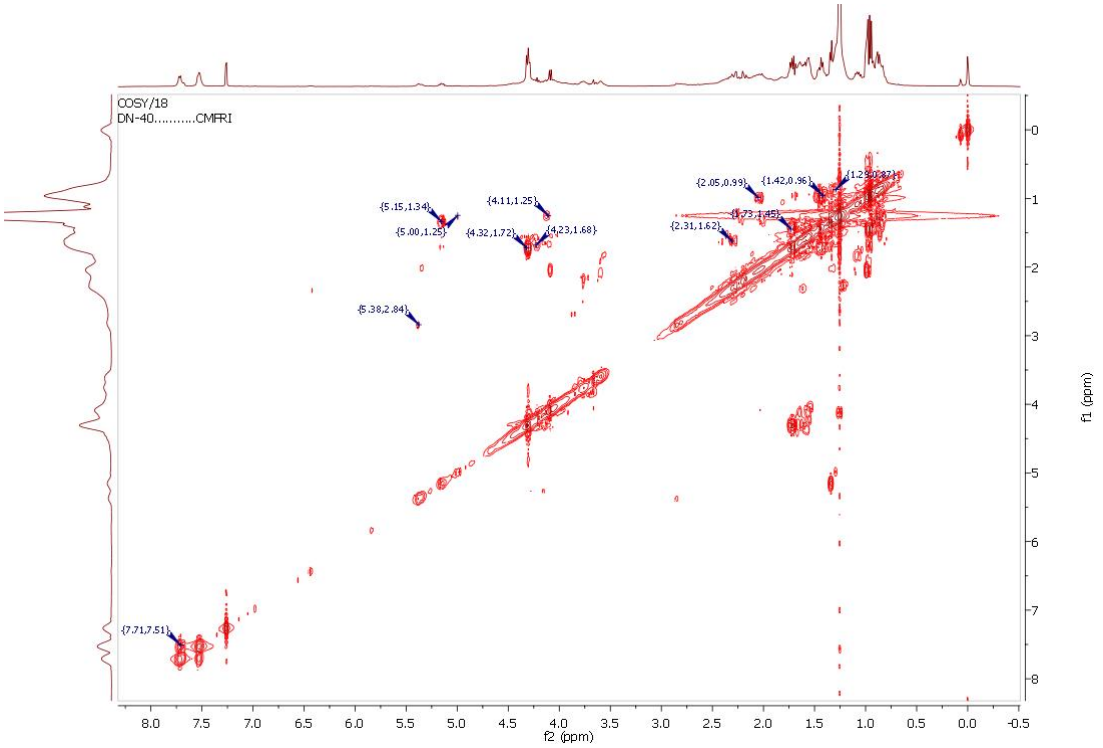


Fig. 8.8.5. ^{1}H—^{1}H COSY spectrum of 2-2-[(4-hydroxybenzoyl) oxy] ethyl-4-methoxy-4-2-[(4-methylpentyl) oxy]-3, 4-dihydro-2H-6-pyranylbutanoic acid

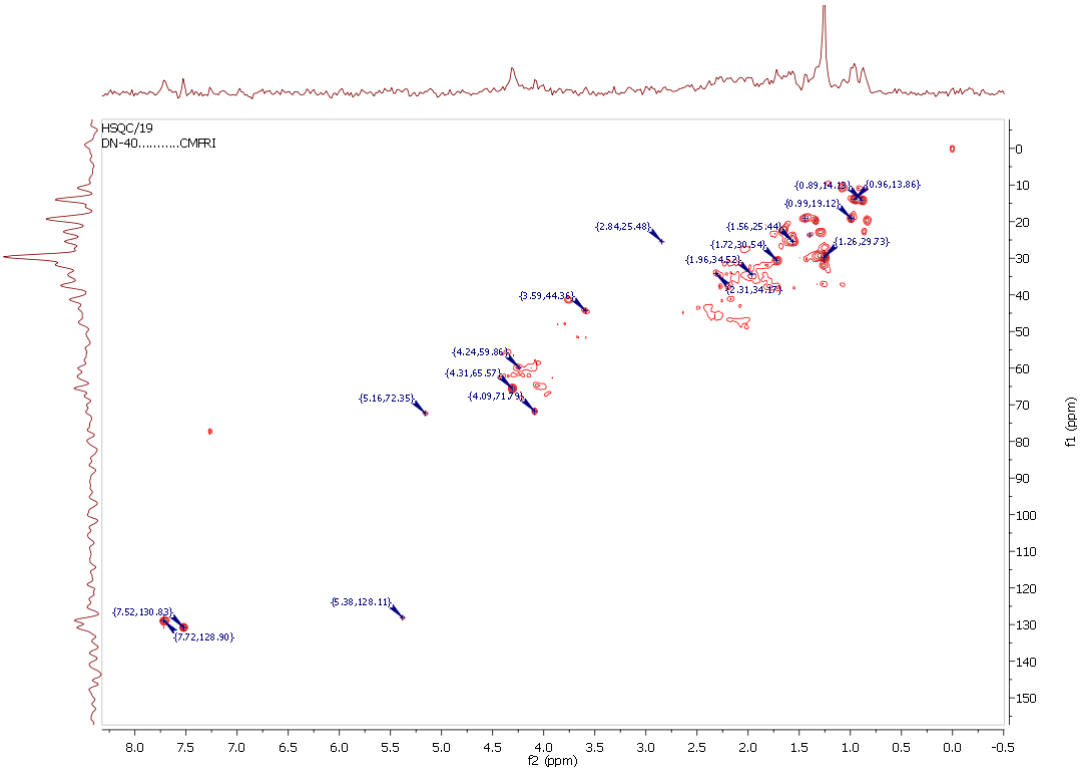


Fig. 8.8.6. HSQC spectrum of 2-2-[(4-hydroxybenzoyl) oxy] ethyl-4-methoxy-4-2-[(4-methylpentyl) oxy]-3, 4-dihydro-2H-6-pyranylbutanoic acid

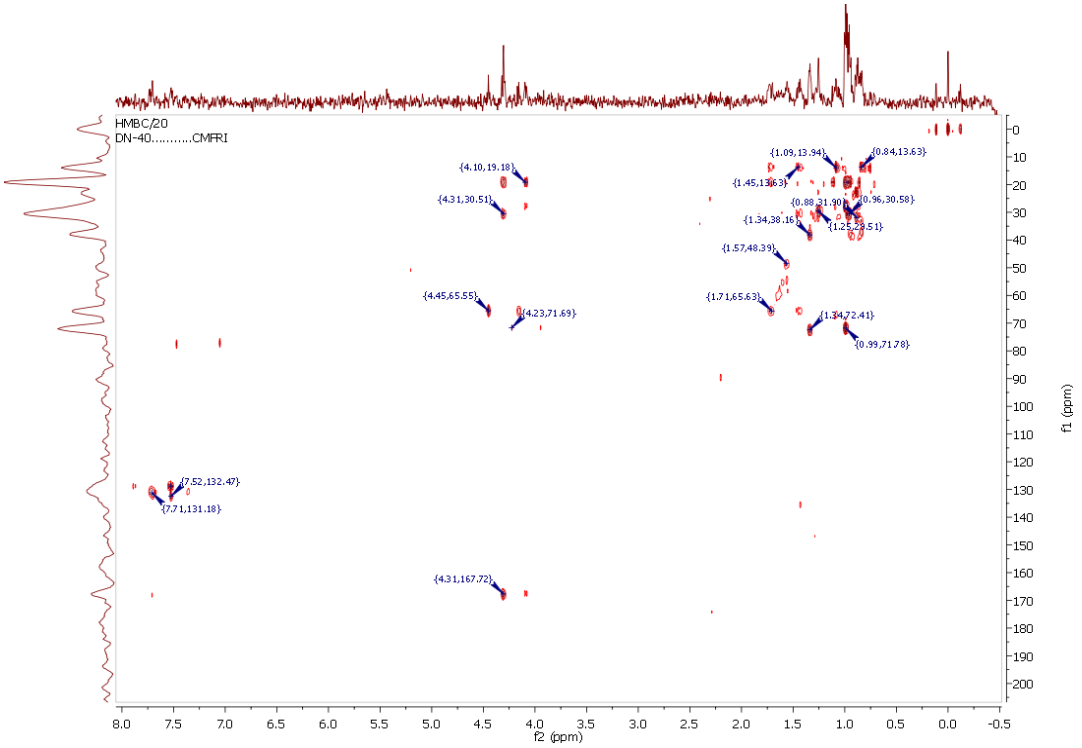


Fig. 8.8.7. HMBC spectrum of 2-2-[(4-hydroxybenzoyl) oxy] ethyl-4-methoxy-4-2-[(4-methylpentyl) oxy]-3, 4-dihydro-2H-6-pyranylbutanoic acid

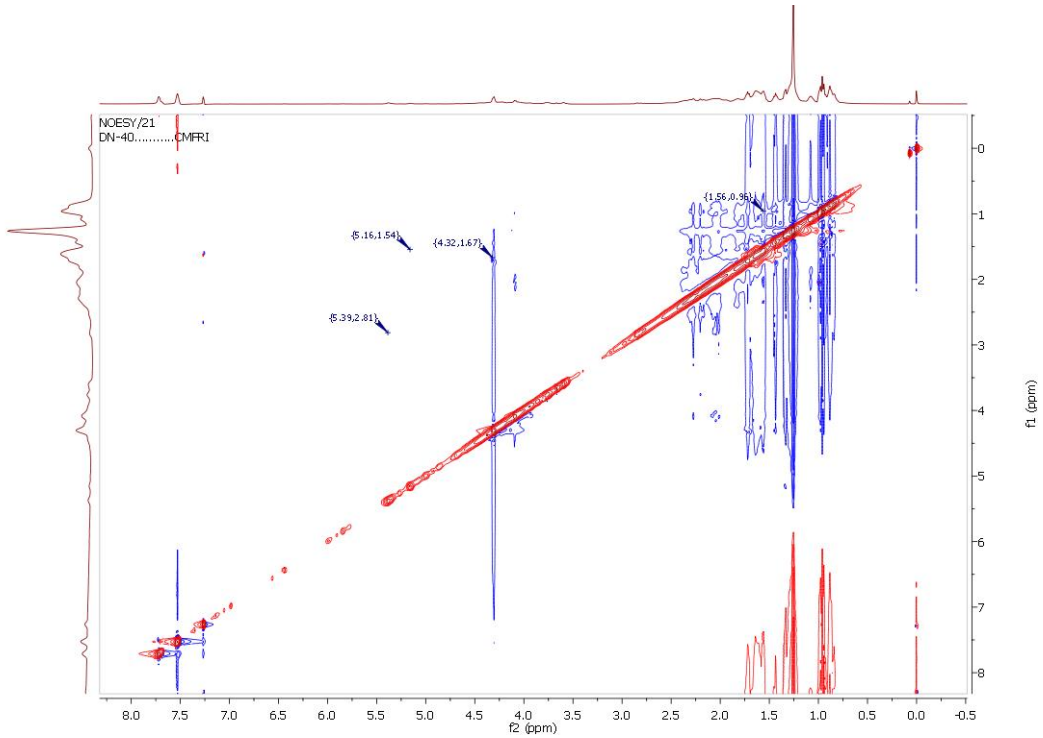


Fig. 8.8.8. NOE spectrum of 2-2-[(4-hydroxybenzoyl) oxy] ethyl-4-methoxy-4-2-[(4-methylpentyl) oxy]-3, 4-dihydro-2H-6-pyranylbutanoic acid

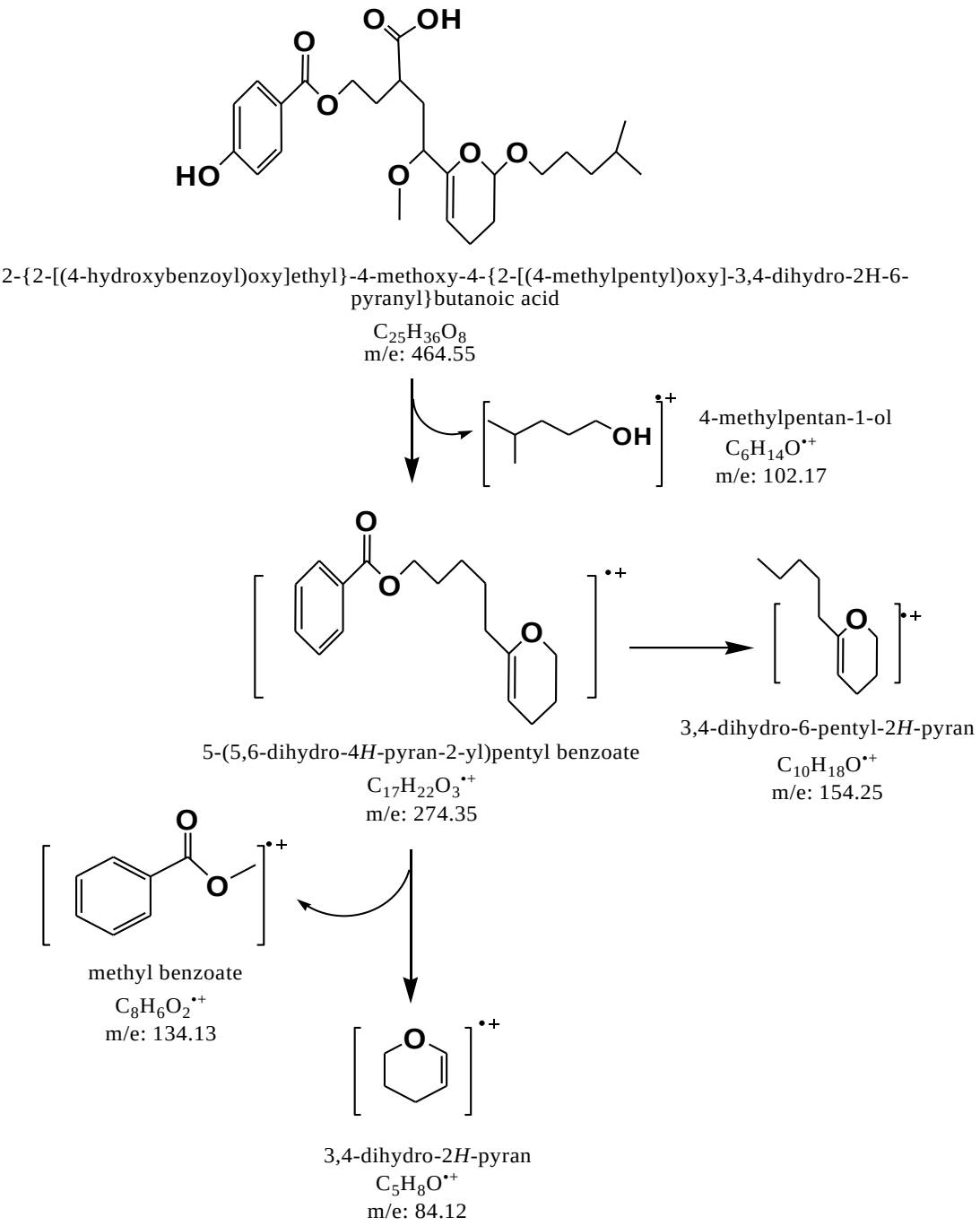


Fig. 8.8.9. Fragmentation pattern of 2-2-[(4-hydroxybenzoyl) oxy] ethyl-4-methoxy-4-2-[(4-methylpentyl) oxy]-3, 4-dihydro-2H-6-pyranylbutanoic acid

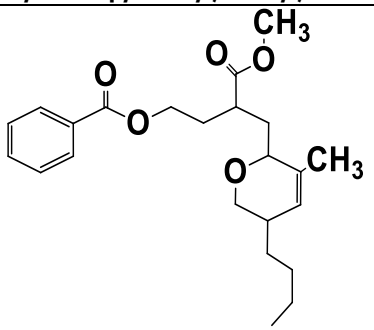
Two styryl-pyran-2-ones, 5, 6-dehydrokawain and 4'-hydroxy-5, 6-dehydrokawain, have been isolated from ethanolic extracts of the seeds of *Alpinia*

blepharocalyx, and the latter has shown significant antiproliferative activity against murine colon 26-L5 carcinoma (ED50: 20.7 μM) and human HT-1080 fibrosarcoma (ED50: 20.1 μM) cells (Ali *et al.,* 2001). Two new dihydrostyrylpyrones and a styrylpyrone, together with a known styrylpyrone, have been isolated from the ethyl acetate-soluble fraction of an aqueous ethanol (1:4) extract of the herb *Polygala sabulosa* (Pizzolatti *et al.,* 2000), which is used as a topical anaesthetic in folk medicine. The 5, 6-Dihydro-2-pyranones, dihydromethysticin and methysticin have been reported from the root extracts of Kava (Goel & Ram 2009). Three new furan and pyran derivatives named aspericins A-C, as well as a known asperic acid, have been isolated from the marine-derived fungus *Rhizopus* sp. These compounds were found to be cytotoxic on P388, A549, HL-60, and BEL-7420 cell lines (Wang *et al.,* 2010). Butyl 2-(4-oxo-5,6-dihydro-2H-pyran-3-yl) acetate, 4-hydroxyphenethyl 2-(4-oxo-5,6-dihydro-2H-pyran-3-yl) acetate, and 3-hydroxybenzyl 2-(4-oxo-5,6-dihydro-2H-pyran-3-yl) acetate have been isolated and characterized from the ethyl acetate extract of the marine fungus Penicillium sp. (Wu *et al.,* 2010). 4-Methoxy pyran isolated from ethanol extracts of the red seaweed *Osmundea pinnatifida* was demonstrated to possess antioxidative and antileishmanial activities (Sabina & Aliya 2011).

8.2.2.3. Structural Characterization of Compound III (HM-50)

Compound III (HM-50)

3-((5-Butyl-3-methyl-5, 6-dihydro-2H-pyran-2-yl) methyl)-4-methoxy-4-oxobutyl benzoate



Yield	81.44 mg (1.02 %*)
Physical description	Pale yellow powder
Molecular formula	$C_{23}H_{32}O_5$
Molecular weight	388.4814

* % Yield based on starting extract

The spectroscopic characterization of the pure and active compound (HM-50), 3-((5-butyl-3-methyl-5, 6-dihydro-2H-pyran-2-yl) methyl)-4-methoxy-4-oxobutyl benzoate are discussed in detail below.

3-((5-Butyl-3-methyl-5,6-dihydro-2H-pyran-2-yl)methyl)-4-methoxy-4-oxobutyl benzoate: Pale yellow powder; UV (MeOH) λ_{max} (log ε): 234 nm (3.81); TLC (Si gel GF_{254} 15 mm; 10% MeOH/EtOAc, v/v); R_f: 0.86; GC (Elite – 5 capillary column 30 m x 0.53 mm i.d.; oven temperature ramp: 60^{o}C for 10 min, rising at 5^{o}C /min to 220^{o}C; 1 ml injection volume/$CHCl_3$) R_t:17.22 min.; Elemental analysis found: C,71.11;H,8.30;O,20.59;R_f: 0.86; R_t: 17.22min.; IR (KBr, cm^{-1}) ν_{max} 731.29 (C-H ρ), 812.06 (aromatic C-H *δ*), 1376.26 (C-H ρ), 1466.91 (C-H *δ*), 1650.12 (C=C ν) 1690.21(C-CO-C ν), 1736.96(C=O ν), 2856.74(C-H ν), 2921.22(C-H ν), 2955.04(C-H ν), 3010.12 (aromatic C-H ν); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.77 - 7.69 (m, 1H), 7.55 (dq, 1H, J=6.8 Hz, 3.4 Hz), 5.38 (d, 1H, J=9.3 Hz), 4.34 (t, 2H, J=6.7 Hz), 4.24 (t, 1H, J=5.9 Hz), 4.11 (d, 2H, J=6.7 Hz), 0.88(t, 3H, J=6.7 Hz), 2.16(s, 3H); ^{13}C-NMR, ^{1}H-^{1}H-COSY and HMBC data (details under the Table 8.7); HRMS (ESI) m/e: 389.5668 calcd. for $C_{23}H_{33}O_5$ 389.4814; found 389.5668 $[M+H]^+$.

HM-50 showed 56.2 % DPPH radical scavenging activity, 21.1 % Fe^{2+} ion chelating activity and 1.34 MDAEQ/kg TBARS formation inhibitory activity (Table 8.4). The ^{1}H NMR in conjugation with ^{13}C-NMR recorded the presence of three methyl signals at δ 3.69, δ 2.16 and δ 0.88. The $–CH_2$- protons appeared at δ 4.11 are due to the presence of the dihydropyran ring system and have been assigned to be present at the C-18 position of the ring structure. Two methylene groups have been assigned to occupy at the C8-9 positions, and the one with δ 4.33 shifted downfield due to the presence of an extended conjugation probably linked to an aromatic moiety. The HMBC correlation of the proton at δ 4.33 with the carbon atom at δ 167.70 apparently indicates the presence of -C=O(O). HSQC and HMBC experiments revealed that the ester group linked to the methylene group at δ 4.33 is attached to the substituted aryl ring. The aromatic protons showed their characteristic signals at δ 7.77. ^{1}H–^{1}H COSY experiments revealed that the protons at δ 4.33 (d) correlate with the methylene protons at δ 1.72 (assigned to be as H-9) and the –CH proton at δ 2.34, the latter is assigned to be attached to a strongly electronegative group. HMBC correlations were apparent between H-10 (δ 2.34) with that of a

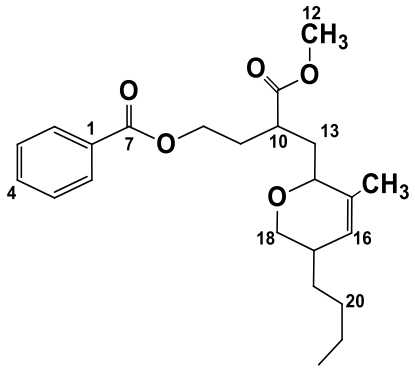
carboxyl carbon at δ 173.30, which apparently indicates the presence of butanoate moiety. A typical signal characteristic of $–OCH_3$ group was apparent at δ 3.69, which enables to deduce the presence of methyl 4-methoxybutanoate moiety in the compound. The carboxymethyl group at the C-11 position of the compound resulted in strong deshielding of the –CH- proton at δ 2.34, and therefore, has been assigned to be present at the C-10 position of the structure. The chemical shift of the protons at δ 4.11, 2.02, 5.38, and 4.24 along with detailed 2D NMR experiments established the presence of 3,6-dihydro-2H-pyran moiety. The methine proton (gives triplet) at δ 4.24 is characteristic of the junction point of the 3, 6-dihydro-2H-pyran moiety with that of the side chain.

The ^{13}C-NMR spectrum of the purified compound in combination with DEPT experiments indicated the occurrence of 23 carbon atoms in the molecule including two carbonyl carbons at δ 173.30 and δ 167.70 and olefinic carbons at δ 129.95 and 114.05 (Table 8.7). The ^{13}C NMR spectrum of the purified compound displayed two quaternary carbons (δ 173.30 and δ 167.70) atoms bearing the carbonyl groups, along with that one bearing the aromatic group (δ 132.45). The low field quaternary signals (^{13}C NMR) is in agreement with that to a quaternary carbon signal carrying the carbonyl groups at C-7 of the structure and this was supported by the relatively downfield shift of the H8 signal (δ 4.34), which referred to a possible oxygenation in its vicinity. The proton and carbon connectivity deduced from ^{1}H–^{1}H COSY, HSQC and HMBC experiments confirmed the C4 framework attached to the dihydro-2H-pyran moiety at the 17th position of the ring structure. The H–H and C–H connectivities apparent in the ^{1}H–^{1}H COSY and HMBC spectra respectively indicate that one of the eight unsaturations was due to the dihydropyran ring framework. In the HMBC spectrum, it was observed that H-17 (δ 2.02)/C-16 (δ 129.95); H14 (δ 4.24)/C-23 (δ 29.70), 18 (δ 71.79) were correlated with each other (Table 8.7). Strong ^{1}H–^{1}H COSY correlations were also apparent between the protons at δ 2.02 (-CH, assigned to be as C-17)/δ 1.52 (-CH_2, assigned to be as C-19)/ δ 1.44 (-CH_2, assigned to be as C-20)/ δ 0.92 (-CH_2, assigned to be as C-21)/ δ 0.88 (-CH_3, assigned to be as C-22) were observed, which along with the results detailed above support the presence of 3-butyl-3,6-dihydro-5-methyl-2H-pyran moiety. The detailed HMBC spectral analyses demonstrating the presence of 3-((5-butyl-3-methyl-5, 6-dihydro-2H-pyran-2-yl) methyl)-4-methoxy-4- oxobutyl benzoate has been

represented under the Table 8.7. The relative stereochemistry of the chiral centres, particularly that of C-10 (with proton chemical shift at δ 2.34 as deduced by HSQC experiments) carrying the –COOMe group of the framework and that of C-14 (bearing the –CH group proton at δ 4.24) and C-17 (bearing the –CH group proton at δ 2.02), was deduced from the NOESY spectrum of the compound and the J-values. NOE couplings were observed between Hα-10 (δ 2.34) /Hα-17 (δ 2.02) thus indicating that these groups must be equatorial and on the α-side of the molecule. The methine proton at C-14 group (bearing the –CH group proton at δ 4.24) did not exhibit NOE interactions with H-17 and H-10, which are at the α-face of the molecule, thereby indicating that H-14 is at the axial disposition. NOESY correlation between 10-H (δ 2.34) and 17-H (δ 2.02) indicates that 14-H (δ 4.24) is at close proximity to the carboxylate ester.

The IR absorption band (in MeOH) bending vibration bands near 1736.96 cm^{-1} denotes the ester carbonyl absorption. The olefinic (C=C) groups have been symbolized by the absorption bands at 1650.12 and 1056 cm^{-1}. The ultraviolet absorbances at λ_{max} (log e) 234 nm (3.81) was assigned to a chromophore with unsaturation. Its mass spectrum exhibited a molecular ion peak at m/e 388 (HRESIMS m/e 389.5668 $[M+H]^+$; D 0.0 amu, cald for $C_{23}H_{33}O_5$, 389.1452, which in combination with its ^1H and ^{13}C NMR data (Table 8.7) indicated the elemental composition of $C_{23}H_{32}O_5$ as 3-((5-butyl-3-methyl-5,6-dihydro-2H-pyran-2-yl)methyl)-4-methoxy-4- oxobutyl benzoate with eight degrees of unsaturation. The molecular ion peak at m/e 388.22 ($C_{23}H_{32}O_5^{\bullet+}$) appeared to undergo elimination of benzoic (methyl carbonic) anhydride ($C_9H_8O_4^{\bullet+}$, m/e 180.04) to yield 3-butyl-5-methyl-6-pentyl-3,6-dihydro-2H-pyran at m/e 224.21 ($C_{15}H_{28}O$), which undergoes intramolecular rearrangement to afford the fragment with m/e 114.10 ($C_7H_{14}O^{\bullet+}$) assigned to be as 3, 5-dimethyltetrahydro-2H-pyran. Appearance of the fragment at m/e 136.05 indicates the presence of methyl benzoate moiety ($C_8H_8O_2^{\bullet+}$), resulted from the molecular ion peak. The base peak was found to be due to the tropylium ion ($C_7H_7^+$, m/e 91.05), whereas peaks of good intensity appeared at m/e 148.02 ($C_8H_4O_3$) apparently due to the phthalic anhydride moiety.

Table 8.7 NMR spectroscopic data of 3-((5-butyl-3-methyl-5,6-dihydro-2H-pyran-2-yl)methyl)-4-methoxy-4-oxobutyl benzoate in $CDCl_3$.[a]



Carbon no.	^{13}C NMR (DEPT)	H	δ^{1}H NMR (int., mult., J in Hz)[b]	^{1}H-^{1}H COSY	HMBC (^{1}H-^{13}C)
1	132.45				
2	130.87	2H	7.77 - 7.69 (m, 1H)	H-3,H-7	C-7,4
3	129.95	3H	7.55 (dq,1H, J=6.8,3.4)		
4	128.80	4H	7.55 (dq,1H, J=6.8,3.4)		
5	128.84	5H	7.55 (dq,1H, J=6.8,3.4)	H-6	
6	130.90	6H	7.77 - 7.69 (m, 1H)		C-5
7	167.70				
8	65.57	8H	4.34 (t,2H,J=6.7)	H-9	C-7,9
9	30.37	9H	1.72(m,2H)	H-10	C-10
10	34.13	10H	2.34(m,1H)	H-13	C-11,13
11	173.30				
12	51.43	12H	3.69(s,3H)		C-11
13	24.80	13H	1.61(m,2H)	H-10, H-14	C-14,9
14	68.16	14H	4.24 (t,1H,J=5.9)		C-18, 23
15	114.05				
			2.16(s,C**H**$_3$)		
16	129.95	16H	5.38 (d,1H,J=9.3)	H-17	
17	38.74	17H	2.02(m,1H)	H-18, H-19	C-16
18	71.79	18H	4.11 (d,2H,J=6.7)		C-17
19	22.69	19H	1.52(m,2H)	H-20	C-20
20	19.16	20H	1.44(m,2H)	H-21	
21	14.05	21H	0.92(m,2H)	H-22	C-17,20
22	10.96	22H	0.88(t,3H,J=6.7)		C-21
23	29.70	23H	2.16(s,3H)		

[a] NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers.

[b] Values in ppm, multiplicity and coupling constants (J¼ Hz) are indicated in parentheses. Assignments were made with the aid of the ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY experiments.

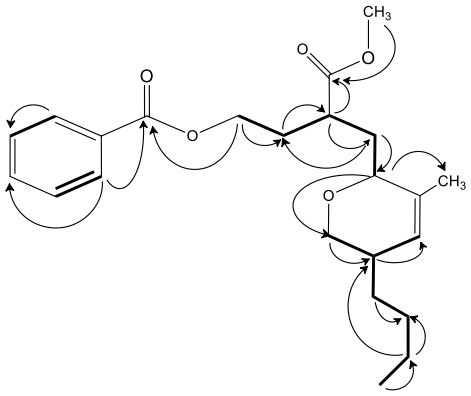


Fig. 8.9.1. ^{1}H—^{1}H COSY and HMBC correlations of 3-((5-butyl-3-methyl-5,6-dihydro-2H-pyran-2-yl)methyl)-4-methoxy-4-oxobutyl benzoate. The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

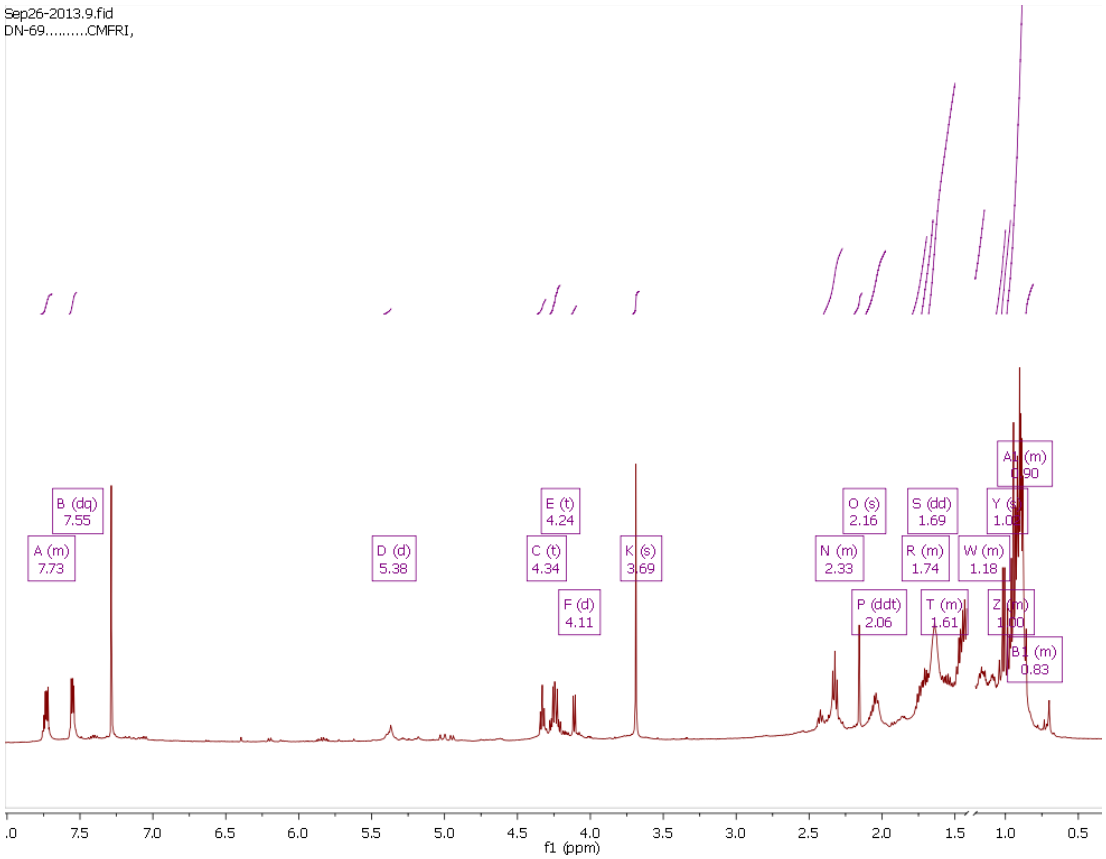


Fig. 8.9.2. Proton NMR spectrum of 3-((5-butyl-3-methyl-5,6-dihydro-2H-pyran-2-yl)methyl)-4-methoxy-4-oxobutyl benzoate

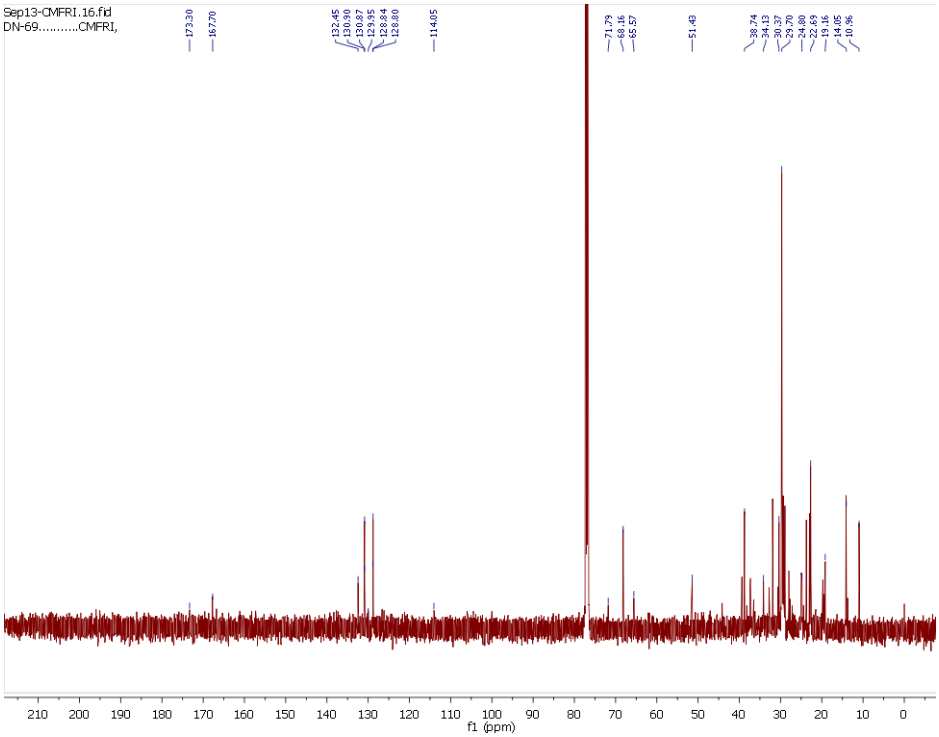


Fig. 8.9.3. ^{13}C NMR spectrum of 3-((5-butyl-3-methyl-5,6-dihydro-2H-pyran-2-yl)methyl)-4-methoxy-4-oxobutyl benzoate

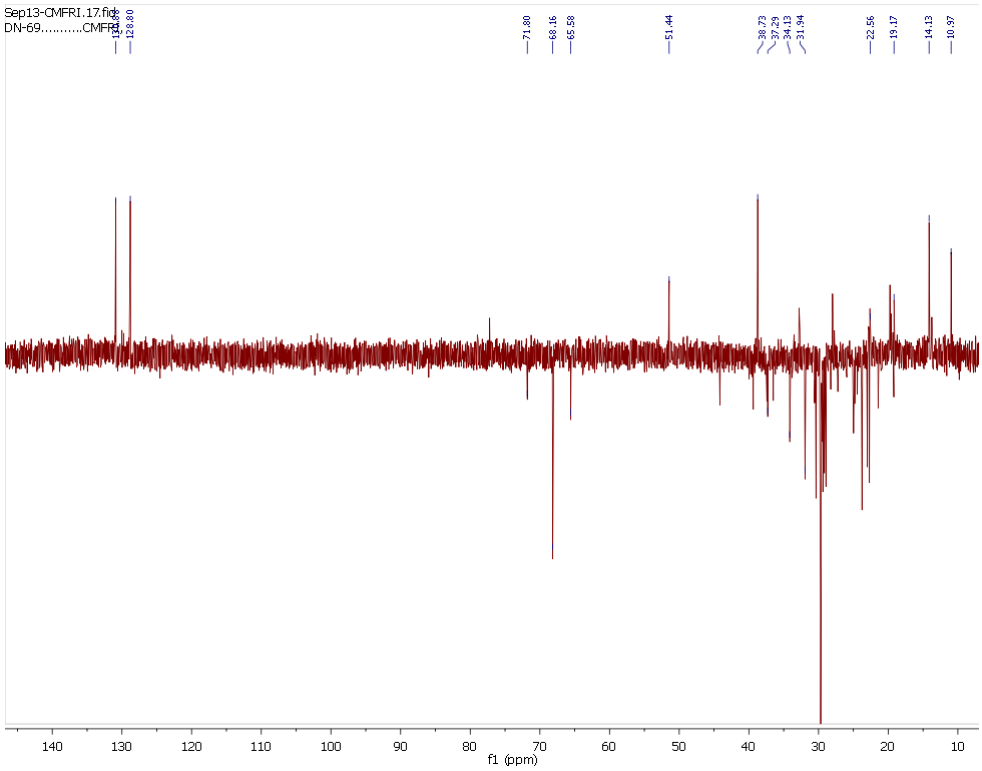


Fig. 8.9.4. DEPT spectrum of 3-((5-butyl-3-methyl-5,6-dihydro-2H-pyran-2-yl)methyl)-4-methoxy-4-oxobutyl benzoate

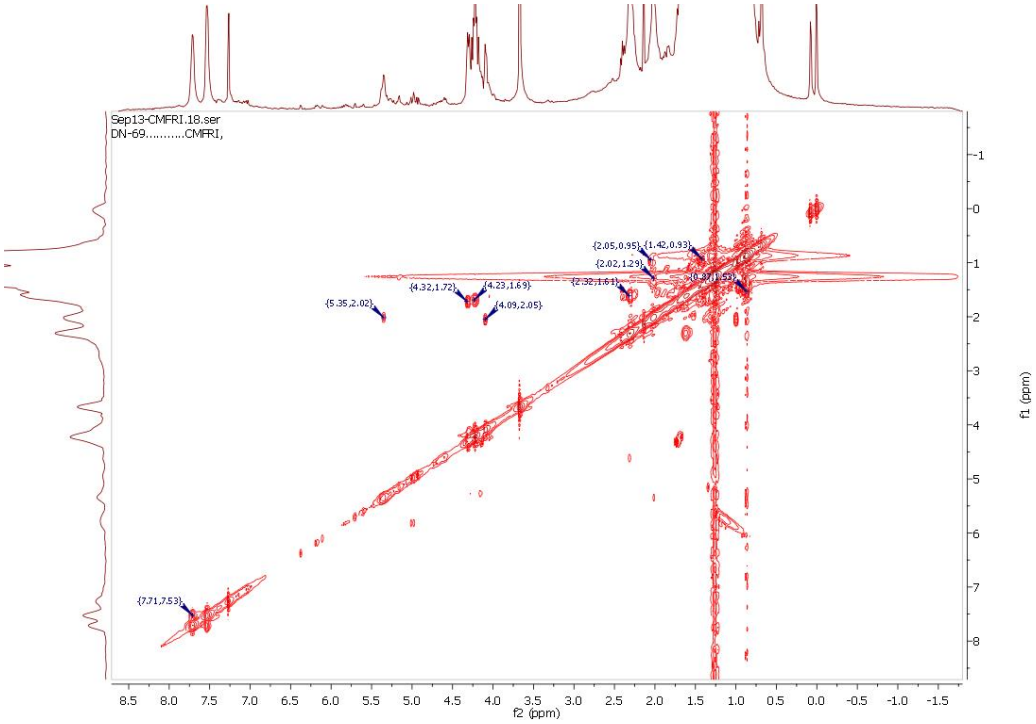


Fig. 8.9.5 ^{1}H–^{1}H COSY spectrum of 3-((5-butyl-3-methyl-5,6-dihydro-2H-pyran-2-yl)methyl)-4-methoxy-4-oxobutyl benzoate

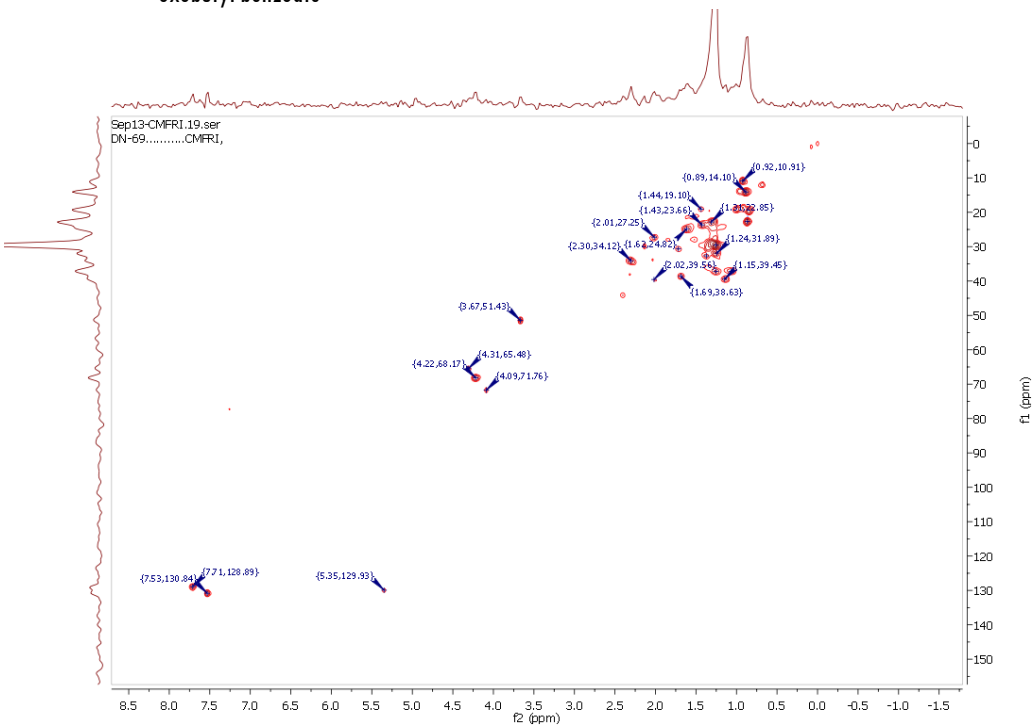


Fig. 8.9.6 HSQC spectrum of 3-((5-butyl-3-methyl-5,6-dihydro-2H-pyran-2-yl)methyl)-4-methoxy-4-oxobutyl benzoate

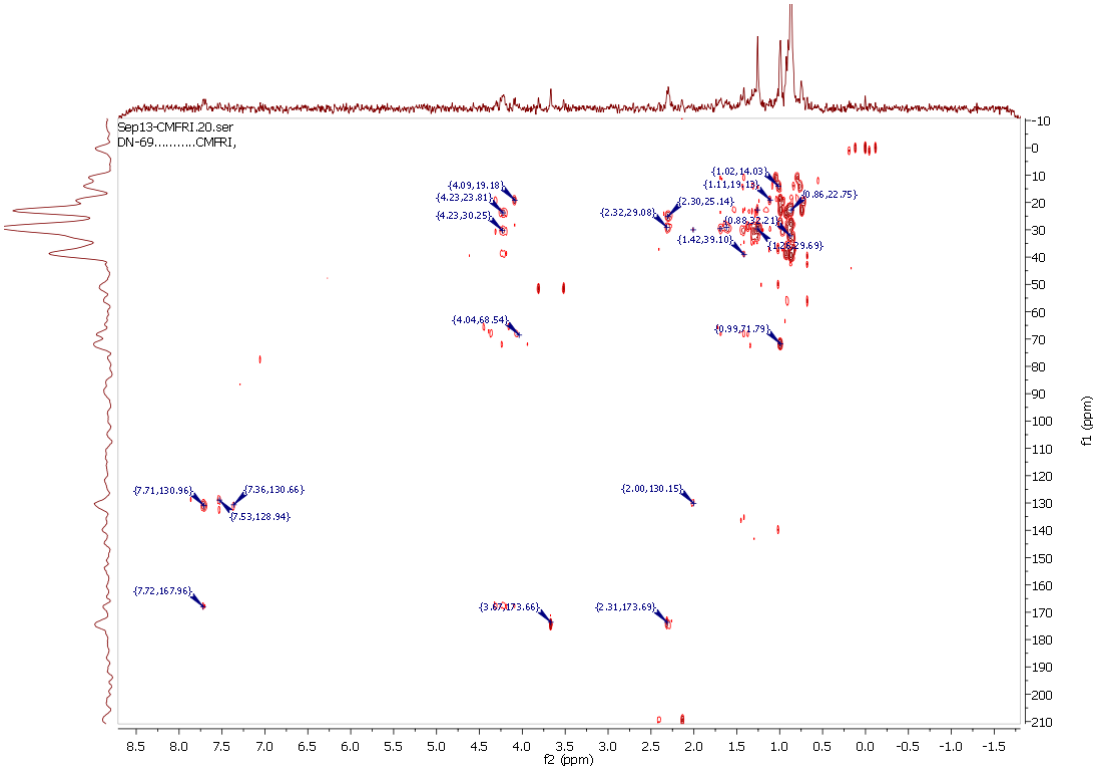


Fig. 8.9.7. HMBC spectrum of 3-((5-butyl-3-methyl-5,6-dihydro-2H-pyran-2-yl)methyl)-4-methoxy-4-oxobutyl benzoate

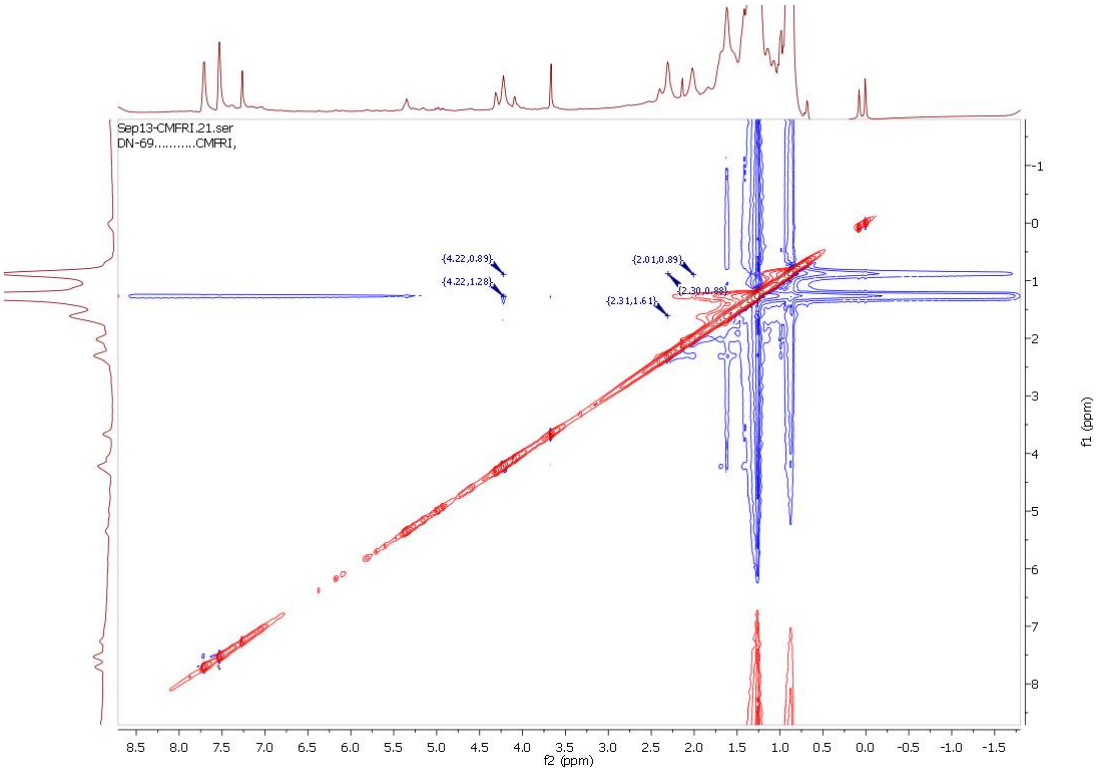


Fig. 8.9.8. NOE spectrum of 3-((5-butyl-3-methyl-5, 6-dihydro-2H-pyran-2-yl) methyl)-4-methoxy-4-oxobutyl benzoate

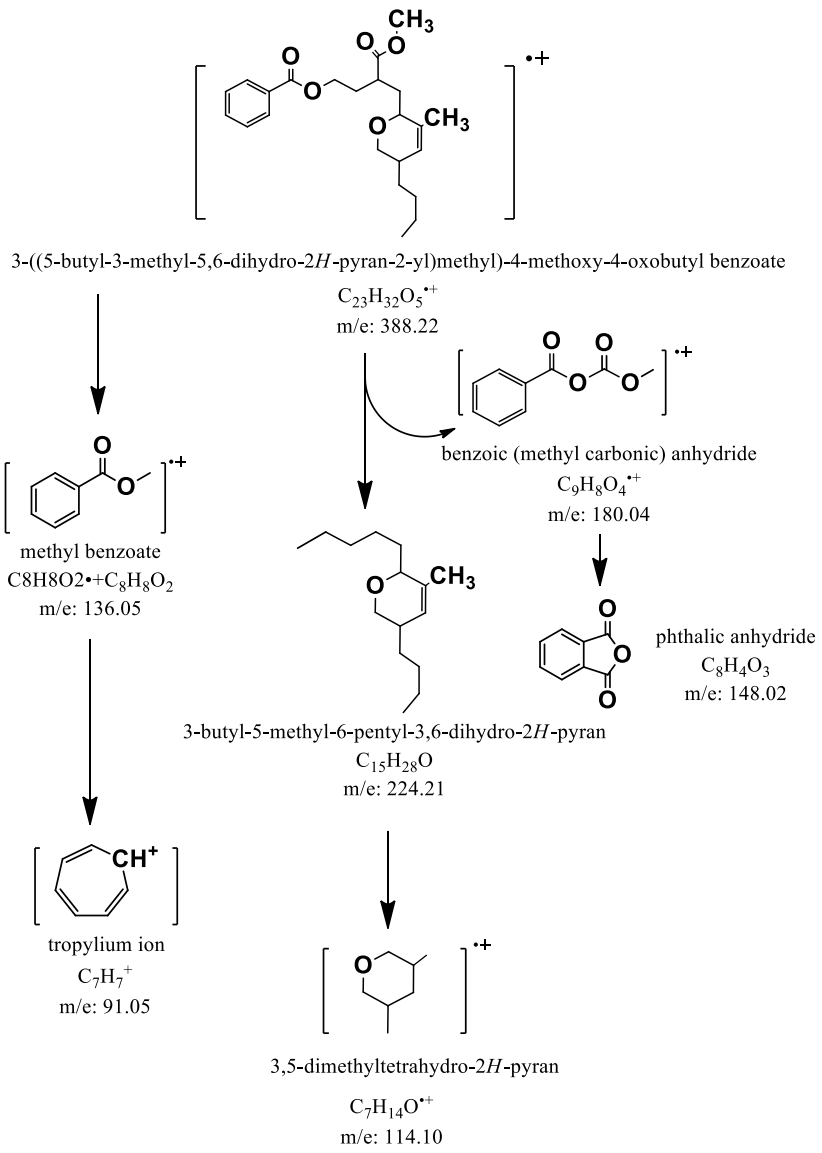


Fig. 8.9.9. Fragmetation pattern of 3-((5-butyl-3-methyl-5, 6-dihydro-2H-pyran-2-yl) methyl)-4-methoxy-4-oxobutyl benzoate

The pyran ring system is widely present in the animal and plant kingdom and possesses diverse pharmacological activities. Compounds with the pyran ring system have been used as precursors for the synthesis of pharmacologically active compounds such as HIV protease inhibitors, antifungals, cardiotonics, anticonvulsants, antimicrobials, and antitumour agents (Goel & Ram 2009). Microbially derived 2-pyranones, obtained from fungi of various genera, are found to display a wide range of cytotoxic, neurotoxic and phytotoxic properties. Earlier

studies reported the isolation of two pyrans, dendroflorin and denchrysan A, from the whole plant of *Dendrobium* genus, used as a health-food (Chen *et al.,* 2008). The bufadienolide class of compounds constitutes the core skeleton of structurally unique 2-pyranone natural products (Deepak *et al.,* 1997) in which a steroid moiety is attached at position five of the lactone ring. This class of compounds is widely used in traditional remedies for the treatment of several ailments, such as infections, rheumatism, inflammation and disorders associated with the central nervous system. Polyhydroxystyryl-pyran-2-one derivative, phelligridin B, together with a new pyranopyrandione, have been isolated from the fruit bodies of the Chinese medicinal fungus *Phellinus igniarius* (Barry *et al.,* 1997). Two new dihydrostyrylpyrones and a styrylpyrone, together with a known styrylpyrone, have been isolated from the ethyl acetate-soluble fraction of an aqueous ethanol (1:4) extract of the herb *Polygala sabulosa*, which is used as a topical anaesthetic in folk medicine (Goel & Ram 2009).

8.2.3. Secondary Metabolites from *Jania rubens*

The yield, antioxidant activities of each column/P-TLC fractions are given in Table 8.8. The R_f of all the P-TLC fractions are also shown in Table 8.8. Among the 9 column fractions obtained from the EtOAc fraction of the MeOH extract, the fractions JR-6 (542.1 mg), JR-8 (789.6 mg) and JR-9 (1012.5) exhibited significantly higher antioxidant activity with respect to scavenge DPPH radicals (81.6, 58.4 & 60.5 %, respectively) ($p<0.05$). Among these fractions, JR-6 and JR-8 showed significantly higher Fe^{2+} ion chelating activity (>54 %) ($p<0.05$).

The further fractionation of JR-6 over p-TLC in 20%EtOAc:*n*-hexane resulted three fractions and a double stage purification of JR-12 through p-TLC in 10%MeOH:$CHCl_3$ yielded the pure compound JR-17 (Compound 1, R_f 0.12). JR-17 showed significantly higher radical scavenging activity (61.4 %) along with high Fe^{2+} ion chelating activity (10.4 %).

The fraction JR-8 was purified using p-TLC (20%EtOAc:*n*-hexane) to get the pure compound JR-23 with low yield (8.5%).

The fraction, JR-9 which exhibited high antioxidant activity was further chromatographed using CC (*n*-hexane/EtOAc/MeOH) to obtain six fractions, JR-24 to JR-29. Though JR-26, JR-28 and JR-29 on subsequent purifications yielded highly active pure compounds, JR-32, JR-39, JR-43 and JR-45 all these compounds gave low yield (< 10 mg).

Table 8.8 Yield, Rf, DPPH radical scavenging activity, lipid peroxidation inhibitory activity (TBARS formation inhibitory activity) and Fe^{2+} ion chelating activity (%) of the fractions obtained by the purification of *Jania rubens*

	Yield(mg)	R_f	DPPH radical scavenging activity	TBARS formation inhibitory activity	Fe^{2+}ion chelating activity
JR EtOAc fraction (CC E/H)					
JR-1 (100% H)	1564.1		31.4	3.16	14.26
JR-2 (1% E/H)	685.5		0	10.96	13.16
JR-3 (5% E/H)	879.4		22.46	10.01	0.26
JR-4 (20% E/H)	654.2		30.43	7.21	4.31
JR-5 (30% E/H)	771.87		20.2	4.31	5.26
JR-6 (40% E/H)	542.1		81.64	3.16	64.31
JR-7 (50% E/H)	820.1		17.6	2.01	17.21
JR-8 (70% E/H)	789.6		58.41	4.16	54.11
JR-9 (100% E)	1012.5		60.53	1.01	6.11
JR-6 (PTLC 2% E/H)					
JR-10	242.2	0.26	46.24	1.24	7.11
JR-11	105.69	0.45	30.14	12.01	2.11
JR-12	187.14	0.71	58.25	6.01	16.12
JR-10 (PTLC 30% E/H)					
JR-13	235	0.72	16.46	1.01	4.31
JR-14	5.01	0.9	62.51	0.56	0.26
JR-12 (PTLC 10% M/C)			14.31	10.01	4.04
JR-15	124.45	0.56	0.41	12.65	5.12
JR-16	56.44	0.95	48.26	0.96	15.13
JR-16 (PTLC 10% E/H)					
JR-17 COMPOUND I	45.65	0.12	91.41	0.81	40.41
JR-18	3.25	0.34	0.1	1.34	0.68
JR-19	2.56	0.55	0	NA	NA
JR-20	2.14	0.86	0	NA	NA
JR-8 (PTLC 20% E/H)					
JR-21	490	0.16	24.21	0.96	4.41
JR-22	284.6	0.24	31.34	0.46	14.31
JR-23	8.54	0.77	52.46	0.11	18.24
JR-9 (CC H/E/M)					
JR-24 (10% E/H)	314.47		6.01	0.96	21.46
JR-25 (30% E/H)	251.31		17.26	4.23	22.43
JR-26 (40% E/H)	97.4		39.41	2.34	11.34
JR-27 (60% E/H)	10.01		6.26	1.01	4.89
JR-28 (100 E)	158.78		47.11	2.01	6.24

JR-29 (50 % M/E)	180.01		58.46	0.11	10.39
JR-26 (PTLC 20% E/H)					
JR-30	28.47	0.26	11.01	2.31	11.29
JR-31	59.69	0.47	9.26	0.32	42.46
JR-32	7.14	0.67	51.31	NA	NA
JR-28 (PTLC 20 % E/H)					
JR-33	31.45	0.27	4.01	3.06	6.21
JR-34	80.02	0.52	0.26	4.24	7.01
JR-35	45.69	0.76	52.4	0.56	16.21
JR-35 (PTLC 30% E/H)					
JR-36	7.56	0.46	0.2	NA	NA
JR-37	8.04	0.56	0.1	NA	NA
JR-38	18.24	0.67	4.4	2.01	0.26
JR-39	4.02	0.82	76.1	NA	NA
JR-40	5.14	0.96	18.24	NA	NA
JR-29 (PTLC 40% E/H)					
JR-41	109.33	0.12	1.31	1.61	16.01
JR-42	59.88	0.26	62.36	0.41	7.24
JR-43	8.47	0.78	58	NA	NA
JR-42 (PTLC 50% E/H)					
JR-44	48.54	0.5	16.2	2.16	0.65
JR-45	9.5	0.9	74.1	NA	NA

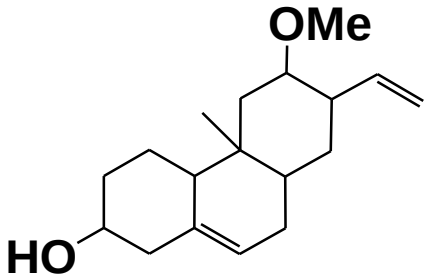
DPPH radical scavenging activity at 0.1 mg/ml is expressed in % (48 h); lipid peroxidation inhibitory activity at 0.1 μg/ml is expressed in MDAEQ/kg sample; Fe^{2+} ion chelating activity at 0.1 mg/ml is expressed in %. NA – NOT ASSAYED ie. the fractions with low yield were evaluated only for DPPH radical scavenging activity. CC – Column chromatography; PTLC – Preparative thin layer chromatography; M – Methanol; E –Ethyl acetate; H – *n*-hexane; C – Chloroform; HM - *Hypnea musciformis*

The results of the chemical investigation of the pure compound isolated from the red seaweed, *Jania rubens* is as follows.

8.2.3.1 Structural Characterization of Compound I (JR -17)

Compound I (JR -17)

6-Methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol



Yield	45.65 mg (0.57 %*)
Physical description	Yellow oil
Molecular formula	$C_{18}H_{28}O_2$
Molecular weight	276.4862

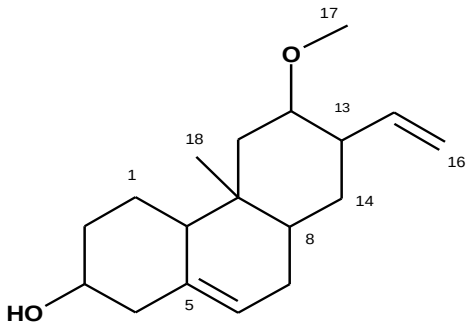
* % yield based on starting EtOAc fraction

6-Methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol: Yellow oil; UV (MeOH) λ_{max} (log ε): 226 nm (3.61); TLC (Si gel GF_{254} 15 mm; 10% EtOAc/*n*-hexane, v/v) R_f: 0.12; GC (Elite – 5 capillary column 30 m x 0.53 mm i.d.; oven temperature ramp: 60°C for 10 min, rising at 5°C /min to 220°C; 1 ml injection volume/$CHCl_3$) R_t: 8.26 min.; IR (KBr, cm^{-1}) ν_{max} 726.29 ρ (C-H), 1240 ν (C-O), 1376.26 ρ (C-H), 1559.50 ν (C=C), 1640 ν (C=C), 1690.21 ν (C-CO-C), 1714 ν (cyclic 6-ring) 2923.22 ν (C-H), 2955.04 ν (C-H alkanes), 3412 ν (O-H); ^{1}H NMR (500 MHz, $CDCl_3$) 5.84(ddt, 1H, J =16.9 Hz), 5.37 (t, J=3.7Hz,1H), 4.99(dd, 2H), 4.33(p, 1H), 4.25(m, 1H), (3.54, OH), 3.69(s, 3H), 1.72(m, 2H), 1.36(m, 2H), 1.13(s, 3H);^{13}C-NMR, ^{1}H-^{1}H-COSY and HMBC data (details under the Table 8.9); HRESIMS *m/e:* 276.4862 ($C_{18}H_{28}O_2$):276.4862 (calcd. for $C_{18}H_{28}O_2$ 276.1244).

Compound **JR-17**, a new derivative of the substituted phenanthrenol (Table 8.9), was isolated as yellowish oil upon repeated chromatography using silica gel as adsorbent. The molecular structures of the purified compound was proposed on the basis of comprehensive analysis of the ^{1}H NMR, ^{13}C NMR, including 2D-NMR experiments (^{1}H–^{1}H–COSY, HSQC, HMBC, and NOESY), and mass spectra. Its mass spectrum exhibited a molecular ion peak at *m/e* 276 (HRESIMS *m/e* 276.4862 $[M]^+$ (calcd. for $C_{18}H_{28}O_2$,276.2224; D 0.0 amu), which in combination with its ^{1}H and ^{13}C-NMR data (Table 8.9) indicated the elemental composition of $C_{18}H_{28}O_2$ as 6-methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol with five degrees of unsaturation containing two double bonds and three ring systems. The molecular ion peak at *m/e* 276.41 appeared to undergo elimination of methanol (CH_3OH, *m/e* 32.04) to yield 1,2,3,4,4a,4b,5,6,7,8,10,10a-dodecahydro-4a-methyl-2-vinylphenanthrene at *m/e* 230.39 ($C_{17}H_{26}^{\bullet+}$), which underwent side chain elimination to afford a fragment with *m/e* 190.32 ($C_{14}H_{22}^{\bullet+}$) (Fig. 8.10.9). Appearance of the fragment at *m/e* 138.25 indicated the presence of decahydronaphthalene moiety ($C_{10}H_{18}$), resulted from the intramolecular rearrangement of 1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydrophenanthrene (Fig. 8.10.9).

The ^{1}H-NMR in conjugation with ^{13}C-NMR recorded the presence of two methyl signals at δ 1.13 and δ 3.69 ppm. The former has been assigned to be due to the methyl group situated at the ring junction, whereas the methyl group at δ 3.69 shifted downfield due to the presence of electronegative group such as oxygen. The methylene group protons at δ 1.72, δ 1.36, and δ 2.36 ppm are assigned to be at C-6 position of the cyclohexane ring structure, and the downfield shift (about δ 1.5 ppm) of the proton at δ 2.36 ppm is due to the presence of the olefinic protons and possibly an electronegative group such as oxygen. Three methylene groups have been assigned to occupy at the C1-4 positions of the ring structure as cyclohexanol moiety.

Table 8.9 NMR spectroscopic data of 6-Methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol in $CDCl_3$.[a]



Carbon no.	^{13}C NMR (DEPT)	H	δ^{1}H NMR (int., mult., J in Hz)[b]	^{1}H-^{1}H COSY	HMBC (^{1}H-^{13}C)
1	30.37	1H	1.36(m,2H)	H-2	
2	28.96	2H	1.72(m,2H)	H-3	
3	72.39	3H	4.33(p, 1H); (3.54,OH)	H-4	C-2,1
4	33.82	4H	2.36(d,2H,J=4.09)		C-5,6
5	133.17				
6	121.02	6H	5.37(t,1H,J=3.7)	H-7	C-7
7	29.67	7H	1.98(m,2H)	H-8	C-14
8	31.93	8H	1.58(m,1H)	H-7	C-14
9	37.14				
10	29.51	10H	1.28(t,1H,J=7.5),		
11	29.16	11H	1.60(m,2H)	H-12	
12	66.27	12H	4.25(m,1H)	H-13	C-17
13	43.12	13H	2.07(m,1H)	H-14, H-15	
14	30.58	14H	1.60(m,2H)	H-8	C-15
15	139.28	15H	5.84(ddt,1H,J=16.9)	H-13, H-16	
16	114.05	16H	4.99(dd,2H)	H-15	C-15,13
17	57.31	17H	3.69(s,3H)		
18	22.69	18H	1.13(s,3H)		C-11

[a] NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers.
[b] Values in ppm, multiplicity and coupling constants (J¼ Hz) are indicated in parentheses. Assignments were made with the aid of the ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY experiments.

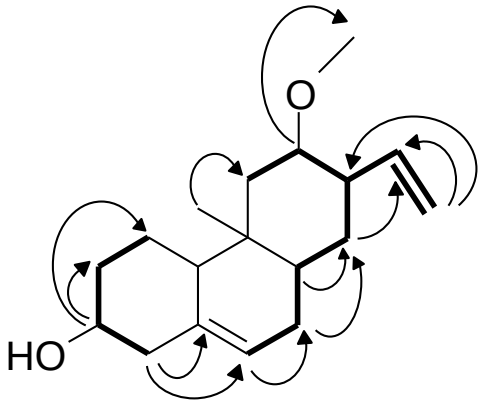


Fig. 8.10.1 ^{1}H–^{1}H COSY and HMBC correlations of 6-Methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol. The key 1H-1H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

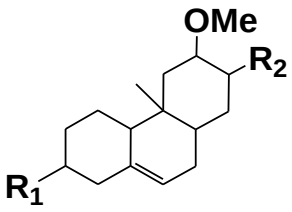
The ^{13}C DEPT-NMR spectrum (Fig. 8.10.4) showed the characteristic -CH_2 signal at the olefinic region (δ114.05 ppm), which showed strong ^{1}H-^{1}H-COSY correlation (Fig. 8.10.1) with the proton situated at δ 5.84 ppm corresponding to the carbon atom at δ 139.28 ppm. This indicated that the olefinic bond is situated external to the ring system. Strong ^{1}H-^{1}H-COSY correlation was apparent 13-H (δ 2.07 ppm) of C-13 (δ 43.12 ppm) and the proton situated at δ 5.84 ppm corresponding to the carbon atom at δ 139.28 ppm, which apparently indicate the presence of vinylcycloalkane moiety. The ^{13}C DEPT-NMR spectrum showed that the carbon at C-5 (δ 133.17 ppm) had no signal in DEPT and HSQC spectra, whereas the carbon at C-6 (δ 121.02 ppm) showed HSQC correlation with the proton at δ 5.37 (t). This indicated that the signature peak of olefinic protons is exocyclic to that of the A ring in the bicyclic system. The ^{13}C NMR spectrum combined with the DEPT spectrum assigned that there were two quaternary carbons, seven each of –CH, and –CH_2–CH_3 carbons in 6-methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol.

Four ^{13}C NMR signals at C-5 (δ 133.17), C-6 (δ 121.02), C-15 (δ 139.28), and C-16 (δ 114.05) indicated the presence of two olefinic bonds. This result has been further confirmed by five degrees of unsaturation with two double bonds and three ring system. The cyclic rings were confirmed by the strong ^{1}H-^{1}H-COSY (Fig. 8.10.1), HSQC and HMBC signals. The carboxyl carbon attached to ring A at C-3 has been confirmed by HMBC correlation. The methoxy carbon at C-17 (δ 57.31) showed HSQC signal at 17-H (δ 3.69, s) situated on C-12 (δ 66.27) as established by the

HMBC correlation with proton at 12-H (δ4.25). The –CH proton attached to the carbon at C-12 (δ 66.27) showed a downfield shift (δ 4.25, m) due to the presence of the electronegative oxygen atom as $–OCH_3$ group (δ 3.69, s). The ring systems A , B and C has been established by strong ^1H-^1H-COSY correlation between 1-H/2-H,2-H/3-H/4-H, 6-H/7-H/8-H, 14-H/13-H, 15-H/16-H, 12-H/11-H. The NOESY experiment showed that the protons 3-H and 6-H are spatially one side, which indicate that the -OH signal at C-3 and the olefinic proton at C-6 (δ 5.37 ppm) are at the same plane.

The protons of the $–CH_2$- group at δ 2.36 are at C-4, and are deshielded due to the presence of the –C=C- moiety at the α-position with respect to the deshielded methylene group. In the $^1H–^1H$ COSY spectrum, couplings were apparent between H-1/H-2, H-3/H-4, which support the presence of cyclohexanol skeleton. The methylene and methine protons showed strong $^1H–^1H$ COSY correlation, which established the presence of octahydronaphthalene ring structure. The –CH proton at δ 2.07 is shifted downfield due to the presence of –C=C- group at its β-position and a α–OH group to assign the structure of 1-methoxy-2-vinylcyclohexane. The methine proton (gives triplet of doublet) at δ 1.58 ppm is characteristic of the junction point of the two rings (C-8). The ^1H-NMR spectrum showed one exchangeable hydroxyl proton at δ 3.54 (1H, bs in $CDCl_3$), which disappeared upon addition of D_2O. The ^{13}C NMR spectrum of the purified compound in combination with DEPT experiments indicated the occurrence of 18 carbon atoms in the molecule including seven methine carbons, one oxygenated methine carbon at δ 72.39 (Table 8.9). The ^{13}C NMR spectrum of the purified compound displayed two quaternary carbon (δ 133.17, 37.14) atoms. The high field quaternary signals (^{13}C NMR) of δ 133.17 are in agreement with that to a quaternary carbon signal carrying the olefinic group at C-5 of the ring structure. The position of the hydroxyl group at C-3 was confirmed from the $^1H–^1H$ COSY, HSQC, NOESY, and HMBC spectra. In the $^1H–^1H$ COSY spectrum, couplings were apparent between H-1/H-2, 3, 4, and H-6/H-7/H-8, H-14/H-13/H-12, which support the presence of phenanthrene skeleton. The point of cyclization of the rings was indicated by the low-field shift of H-10 at δ 1.28, which has been coupled to the H-18 methyl group at δ 1.13 (d, J = 6.0 Hz). Also, the $^1H–^1H$ COSY correlations between H-13/H-14/H-15 supports the presence of the terminal propene moiety at the 1-methoxy-2-

vinylcyclohexane ring system. The proton and carbon connectivity deduced from HSQC and HMBC experiments confirmed the dodecahydro-4b-methylphenanthren-2-ol attached to the side chain 1-propene moiety at the 13th position of the C ring. In the HMBC spectrum, it was observed that H-3/C-2; H-4/C-5,; H-8/C-14 and H-14/C-15 were correlated with each other (Table 8.9). The HMBC analysis revealed that the carbon at δ 72.39 (C-3), bearing the hydroxyl group, correlated with methylene proton δ 1.36 (H-1). Placement of the propene group at the C-13 was confirmed by the downfield shift of methine proton H-13 (δ 2.07) in the compound as compared to the baseline value and also by the long-range correlation of H-13 to the olefinic carbon at δ 114.05 in the HMBC spectrum (Table 8.9). The geometric isomerisms of the olefinic protons have been established by the 1H coupling constants suitable for the geometries (Z and E form). The relative stereochemistry of the chiral centres, particularly that of C-3 and 12 carrying the hydroxyl and methoxyl groups of the phenanthrene ring framework, was deduced from the NOESY spectrum of the compound and the J-values. NOE couplings were observed between Hα-6 (δ 5.37) / Hα-15 (δ 5.84) thus indicating that these groups must be equatorial and on the α-side of the molecule. The methine proton at C-3 group did not exhibit NOE interactions with Hα-6/Hα-15, which is at the α-face of the molecule, thereby indicating that H-3 is at the axial disposition. An interaction through space of the hydroxyl protons at C-3 (δ 4.33, 1H) and C-12 (δ 4.15, 1H, bs) is only possible for the β-orientation of the hydroxyl group on C-3 and methine proton (δ 4.15, 1H) at C-12. The stereochemistry at the tertiary alcohol at C-3 position was established by the fact that H-3 has mutual NOE correlations with H-12, which had shown to be in the β-face of the molecule (axial configuration) under observation, and has no NOE interactions with H-6 and H-16 in the molecule. This is only possible if the C-3 hydroxyl group lies on the β-side of the molecule.



R_1 = H, -OH
R_2 = H, -CH=CH_2

The 1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol group was demonstrated to be the basic moiety of the antioxidant compound. The presence of vinylcyclohexane moiety and the substitution of the vinyl group at the 7 carbon position have been confirmed by detailed NMR and mass spectroscopic experiments. Other compounds from *Jania rubens* with no vinylcyclohexane group (such as 4a-methyl-1,2,3,4,4a,4b,5,6,7,8,10,10a-dodecahydro-3-phenanthrenyl methyl ether) isolated from this species did not show equivalent antioxidative activity proving that the olefinic group attached to the 1-methoxycyclohexane moiety has major role to scavenge the free radicals species responsible for oxidation activity.

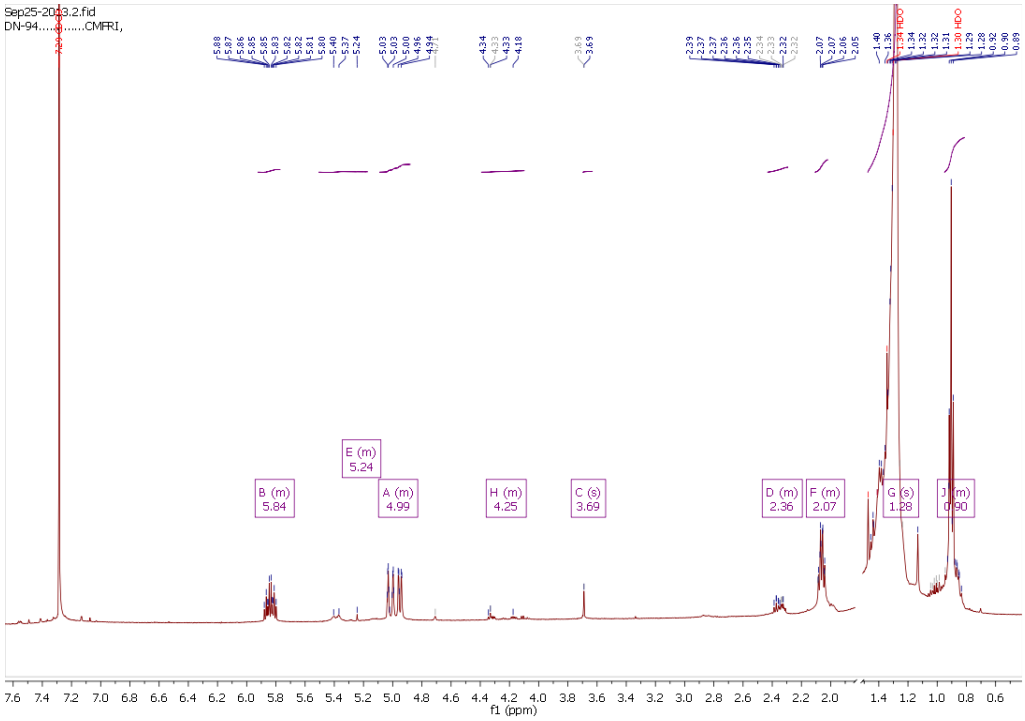


Fig. 8.10.2. Proton NMR spectrum of 6-methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol

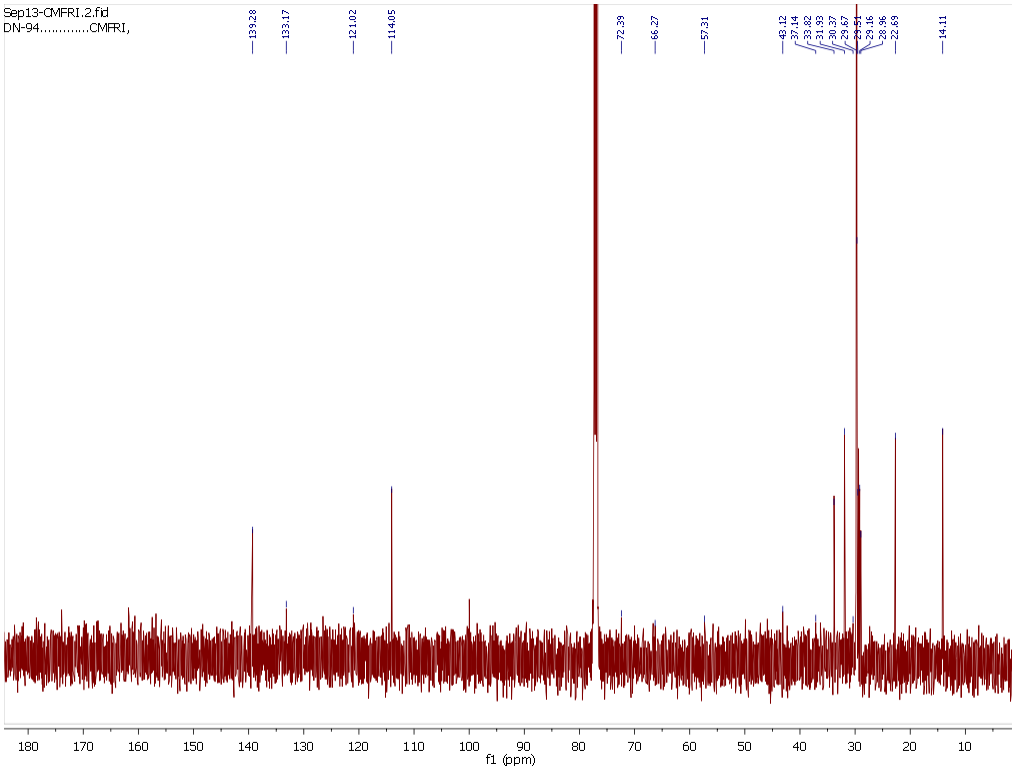


Fig. 8.10.3. ^{13}C NMR spectrum of 6-methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol

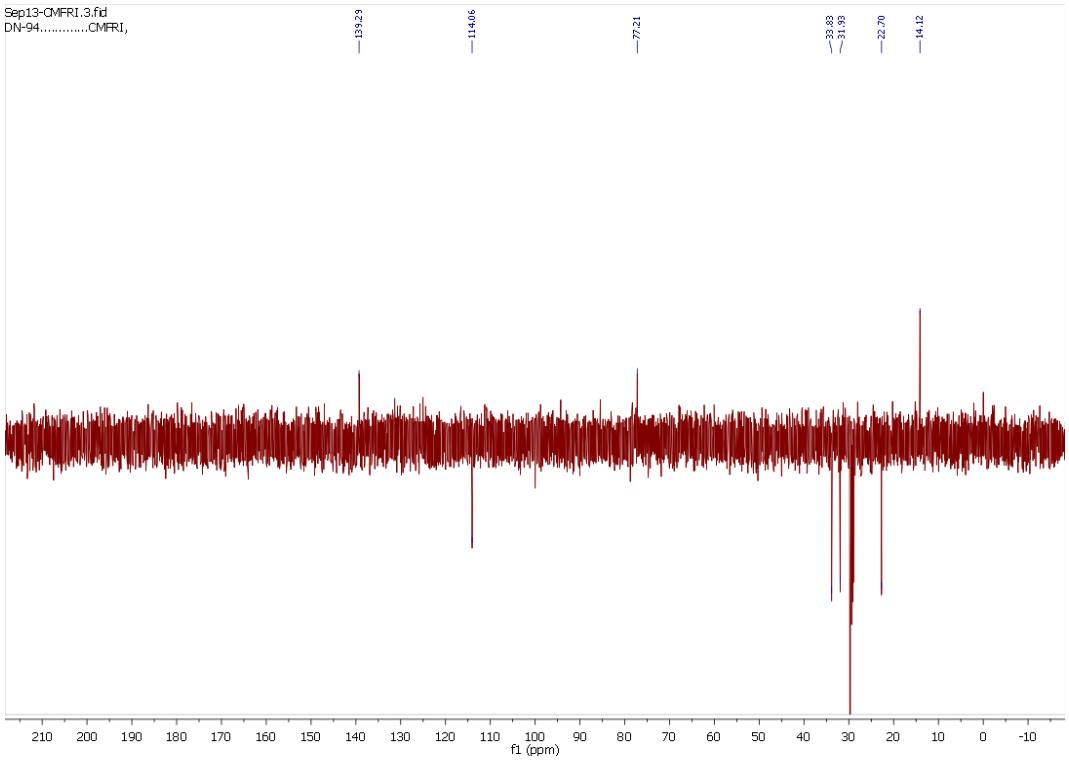


Fig. 8.10.4. DEPT spectrum of 6-methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol

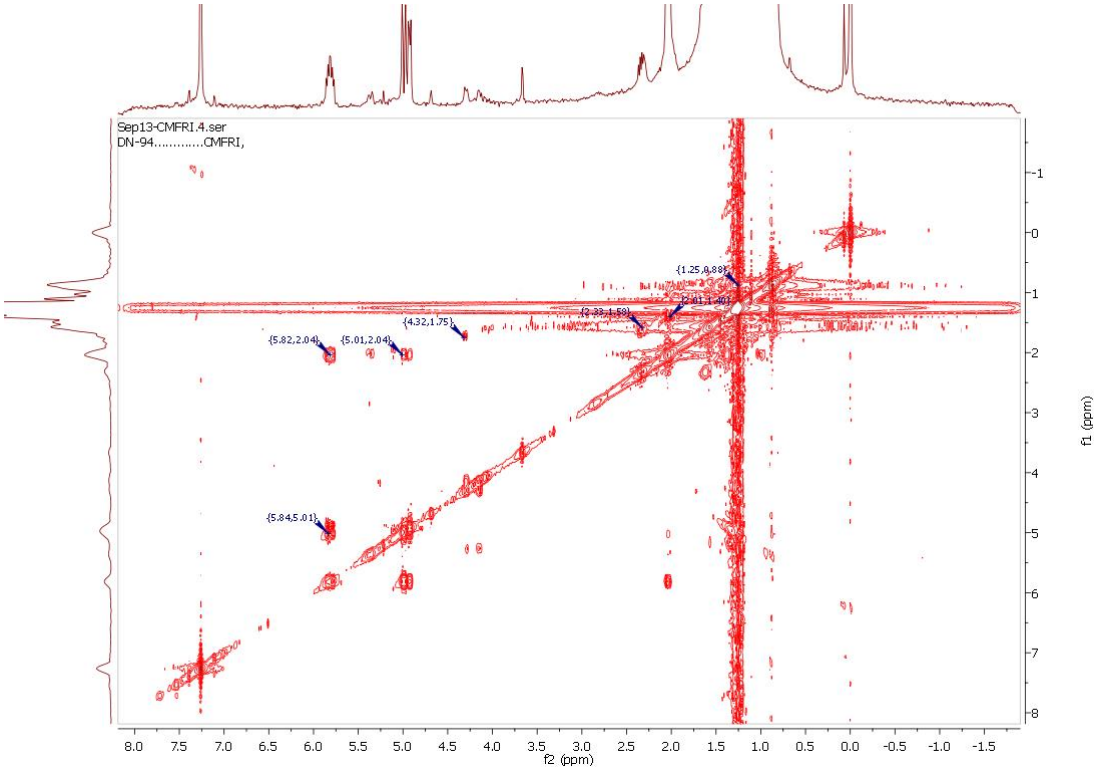


Fig. 8.10.5. ^{1}H–^{1}H COSY spectrum of 6-methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol

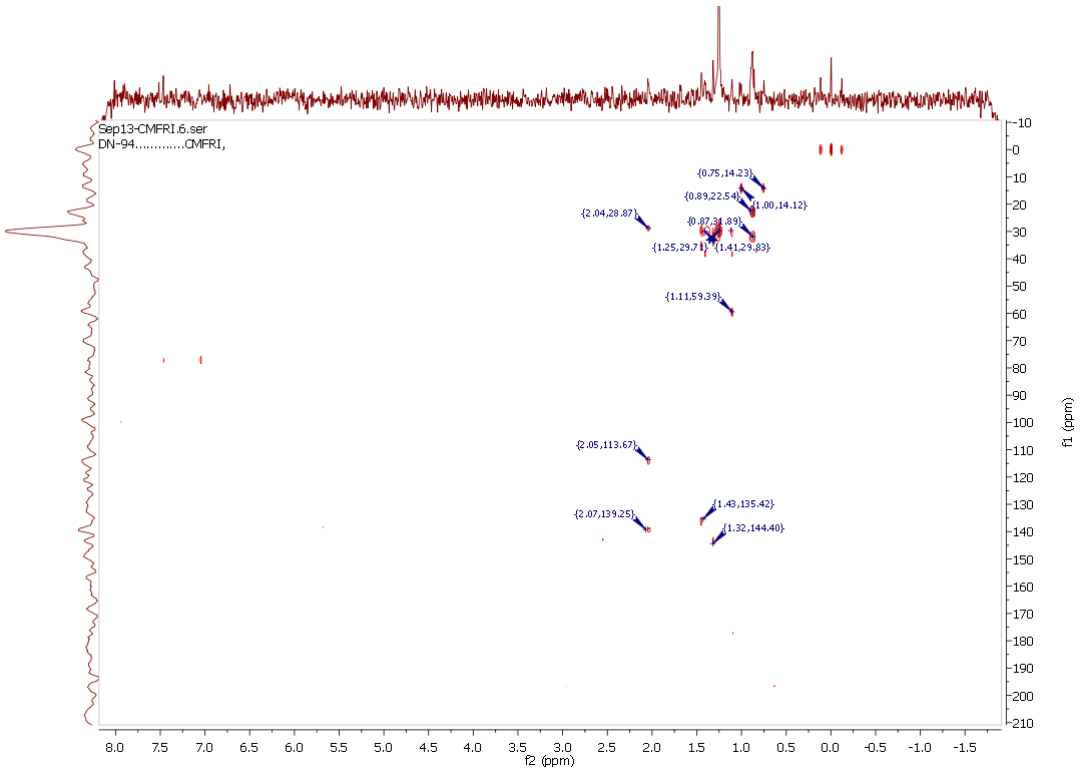


Fig. 8.10.6. HMBC spectrum of 6-Methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol

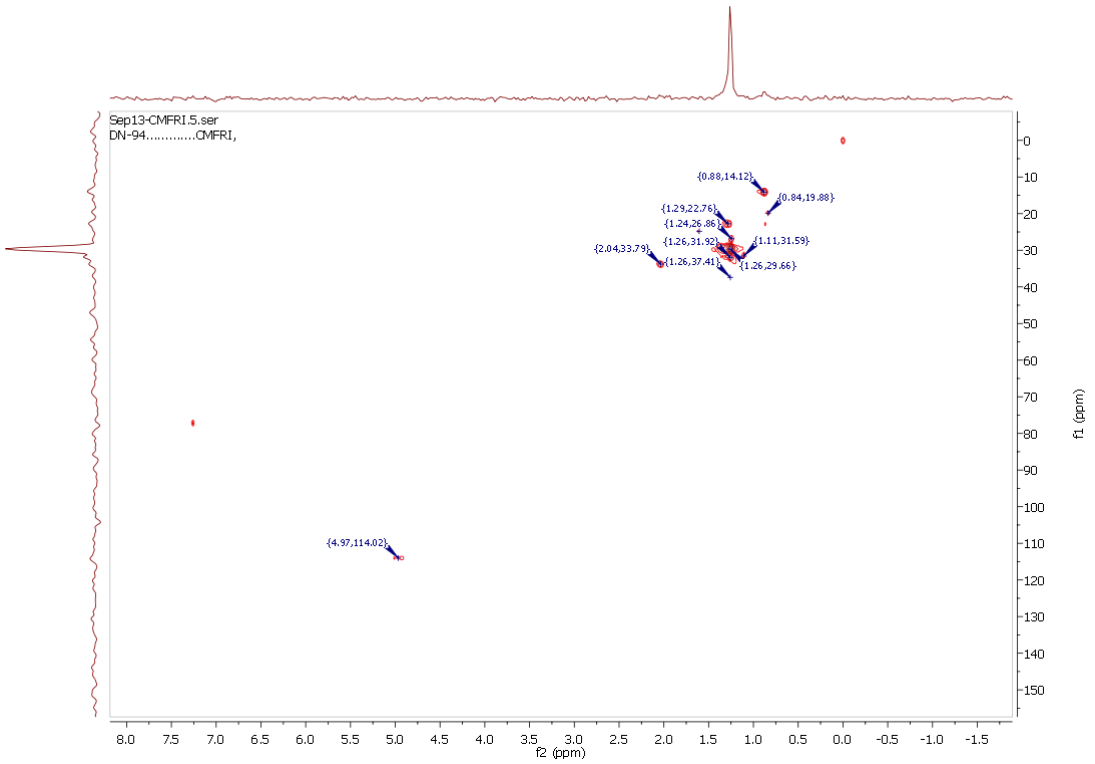


Fig. 8.10.7. HSQC spectrum of 6-Methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol

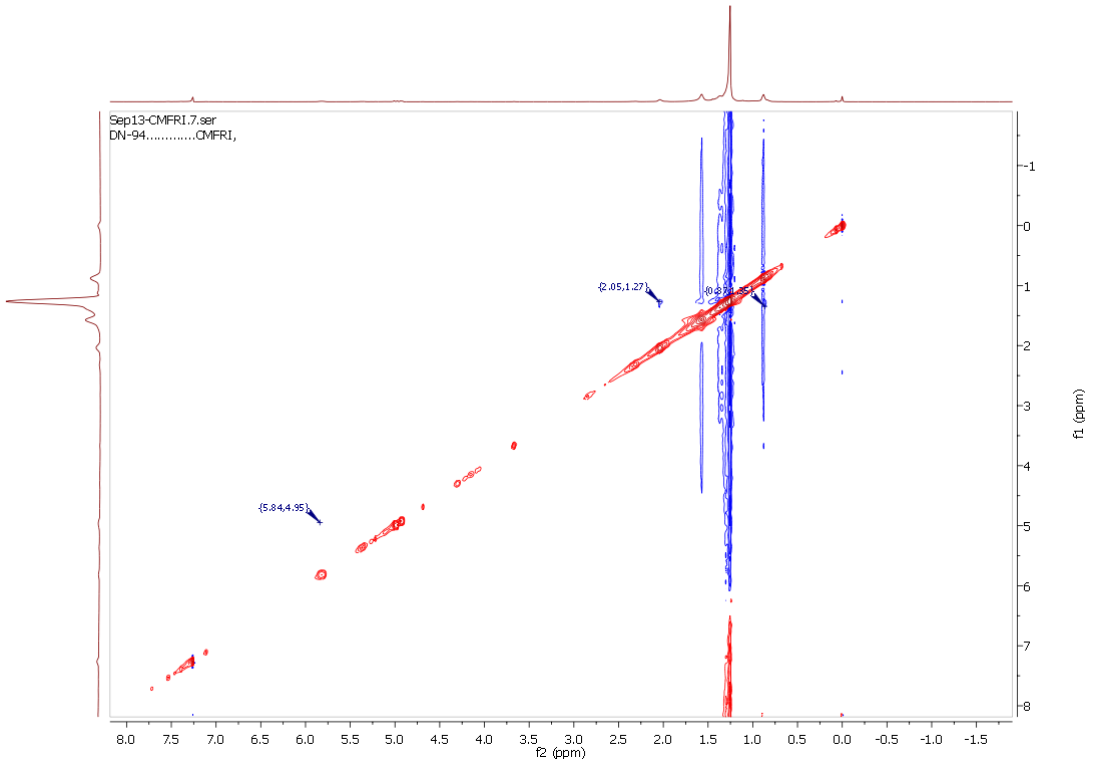
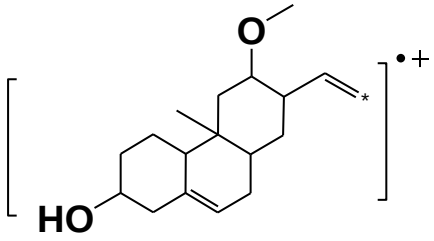


Fig. 8.10.8. NOE spectrum of 6-methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol



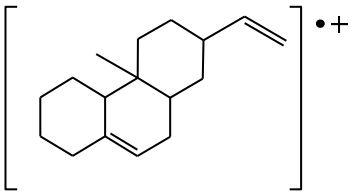
6-Methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol

$C_{18}H_{28}O_2$

m/e: 276.41

CH_3OH

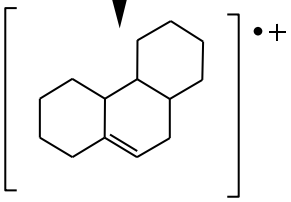
m/e: 32.04



1,2,3,4,4a,4b,5,6,7,8,10,10a-dodecahydro-4a-methyl-2-vinylphenanthrene

$C_{17}H_{26}^{\bullet+}$

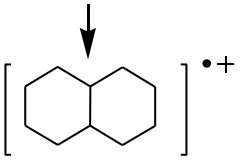
m/e: 230.39



1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydrophenanthrene

$C_{14}H_{22}^{\bullet+}$

m/e: 190.32



decahydronaphthalene

$C_{10}H_{18}$

m/e: 138.25

Fig. 8.10.9. Fragmemtation pattern of 6-methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol

-Phenanthrene and their hydroxyl derivatives isolated from the natural sources were found to be the potential bioactive compounds with pluralities of activities such as cytotoxicity, antimicrobial, spasmolytic, anti-inflammatory, antiplatelet aggregation, antiallergic activities (Fieser 1936; Kovács *et al.,* 2008). Hydroxy or/and methoxy-substituted 9, 10-dihydro/phenanthrenes, methylated, prenylated and other monomeric derivatives, dimeric and trimeric phenanthrenes were found be biologically active compounds. A fairly large number of phenanthrenes have been reported from the species *Dendrobium*, *Bulbophyllum*, and *Epidendrum* (Kovács *et al.,* 2008). These plants have often been used in traditional medicine, and phenanthrenes have therefore been studied for their cytotoxicity, antimicrobial, spasmolytic, anti-inflammatory, antiplatelet aggregation, antiallergic activities and phytotoxicity.

8.3. Conclusions

Currently, reactive oxygen species and lipid oxidation in food industry are being controlled or minimised by the addition of synthetic antioxidants. However, with safety concerns about synthetic antioxidants, considerable interest has arisen in finding alternative sources of antioxidants for use in food systems. The EtOAc fractions from the MeOH extract of *Kappaphycus alvarezii*, *Hypnea muscifomis* and *Jania rubens* was found to be the most effective free radical scavenger (*Chapter 6*), which was fractionated chromatographically to yield different compounds with antioxidative activities. The compounds with potential antioxidative activity isolated from *Kappaphycus alvarezii* are (8Z)-3-Ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione which possessed 81.1 % DPPH radical scavenging activity, 0.99 TBARS formation inhibitory activity and 3.11 % Fe^{2+} ion chelating activity. Another compound, (2Z, 7Z) - methyl 2-ethyl-9-oxo-5-vinyl-1, 4, 5, 5a, 6, 9, 10, 10a-octahydro-1- heptalenecarboxylate., isolated from *K. alvarezii* showed 69.1 % DPPH radical scavenging activity, 0.11 MDAEQ/kg TBARS formation inhibitory activity and 41.0 % Fe^{2+} ion chelating activity.

The compounds isolated and characterized from *Hypnea muscifomis* are 2-(Tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl)ethyl 4-hydroxybenzoate, 2-2-

[(4-hydroxybenzoyl)oxy]ethyl-4-methoxy-4-2-[(4-methylpentyl)oxy]-3,4-dihydro-2H-6-pyranylbutanoic acid, and 3-((5-butyl-3-methyl-5, 6-dihydro-2H-pyran-2-yl) methyl)-4-methoxy-4-oxobutyl benzoate which possessed > 49 % DPPH radical scavenging activity. Among these 2-[4-butyl-5-(4-hydroxyphenyl) tetrahydro-3-furanyl] ethyl 4-hydroxybenzoate showed high Fe^{2+} ion chelating activity (40.2 %).

The compound 6-methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol isolated from the red seaweed *Jania rubens* was found to possess potential antioxidative properties with 91.4 % DPPH radical scavenging activity, 0.88 MDAEQ/kg TBARS formation inhibitory activity and 40.4 % Fe^{2+} ion chelating activity. The chemical structures of the pure compounds isolated with higher yield, as well as their relative stereochemistries, were established by means of spectral data analyses, including 2D-NMR experiments.

The effective scavenging activity against DPPH radicals confirmed that these pure compounds may have potential as natural antioxidant lead molecules in the food industry. These antioxidative compounds derived from the candidate seaweed species may also contribute towards the scavenging of the free radicals resulting in higher shelf-life of the marine oil/esters with multiple olefinic double bond, which are susceptible towards free radical induced oxidation reactions.

Chapter 9

PREPARATION OF CEPHALOPOD EXTRACT FOR THE ENRICHMENT OF PURIFIED SARDINE OIL

Contents

Background

The *n*-3 PUFAs affect diverse physiological processes including cognitive functions and visual acuity, immunosuppressive and anti-inflammatory actions, anti-thrombotic and anti-arrhythmia activities along with several other activities. As a whole, the intake of *n*- 3 fatty acids has been linked to promotion of human health to fight against numerous diseases. However, the various methods for the purification of these *n*-3 fatty acids may reduce their bioactive properties due to various factors associated with the sequence of purification process. These can effectively be compensated by supplementing these fatty acid concentrates with other marine derived bioactive components. The marine environment comprises complex ecosystems and many of the organisms are known to possess bioactive components as a common means of self-defense or for the protection of eggs and embryos. Hence, this chapter focused to find a component that can enrich the property of the sardine purified methyl esters (PO *Chapter 5*) targeting the anti-inflammatory property.

Marine organisms proved to be rich source of bioactive compounds, which have positive influence on human health and have opened a new perspective for pharmacological development. Cephalopods constitute a major share of marine fauna, and were reported to possess structurally diverse anti-stress metabolites with respect to bioactive properties (Chandran *et al.,* 2009; Chakraborty *et al.,* 2010). Even though many bioactive compounds has been isolated from marine animals, much of the potential compounds from marine animals still remain unharnessed especially the organisms belonging to Class Cephalopoda of Phylum Mollusca. Chemical compounds with oxidation-inhibiting and anti-inflammatory properties are

present in cephalopods as a protective mechanism against various stress factors in oceanic ecosystem (Chakraborty *et al.,* 2010; Kim *et al*., 2008). Absence of oxidative damage in the stress-induced biochemical parameters of mollusks/cephalopods from coastal ecosystem suggested that their cells are equipped with powerful anti-stress secondary metabolites with antioxidative and anti-inflammatory activities, which provide competitive advantages against various oxidative stress factors leading to the development of harmful reactive oxygen species. These sessile species are the richest natural sources of bioactive compounds, many of which belong to novel chemical classes not found in terrestrial sources.

There are limited works to establish the bioactive potential of the Cephalopods. In particular, the cephalopod species belonging to *Loligo duvauceli* (Orbigny, 1848), *Cistopus indicus* (Rapp 1835), *Sepia officinalis* (Linnaeus, 1758) and *Octopus membranaceus* (Quoy & Gaimard 1832) are abundantly available throughout the coastline of South India. It is, therefore, imperative that a systematic search for the development of new sources of bioactive pharmacophores from these edible cephalopods from coastal and marine ecosystem will be helpful for development of bioactive supplements for use as additives in the PUFA concentrates. The present study therefore aims to isolate and characterize the novel bioactive lead molecules from these species of the class Cephalopoda.

9.1. Materials and Methods

9.1.1. Chemicals, Reagents and Instrumentation

Potassium phosphate, Tween 20, *leuco*-2, 7-dichlorofluorescein diacetate, hematin, phenol, arachidonic acid, Lipoxidase extra pure (LOX-5), Cyclooxygenase 1 (from sheep, COX-1), Cycloxygenase-2 (human recombinant, COX-2), and carrageenan were procured from Sigma-Aldrich Chemical Co. Inc. (St. Louis, MO).

An electronic micrometer (aerospace; 0-25 mm range, least count: 0.001 mm) was used for measuring the paw thickness of mice. A rotary vacuum evaporator (Heidolf, Germany) was used for evaporating the solvents.

9.1.2. Samples and Study Area

The cephalopods used in the present study (Fig. 9.1) as follows,

- *Loligo duvauceli* (Orbigny, 1848 [Family - Loliginidae; Order - Myopsida; Common name - Indian Squid],
- *Cistopus indicus* (Rapp, 1835) [Family - Octopodidae; Order - Octopoda; Common name - Old woman Octopus],
- *Sepia officinalis* (Linnaeus, 1758) [Family - Sepiidae; Order - Sepiida; Common name - common cuttlefish],
- *Octopus membranaceus* (Quoy & Gaimard 1832) [Family - Octopodidae; Order - Octopoda].

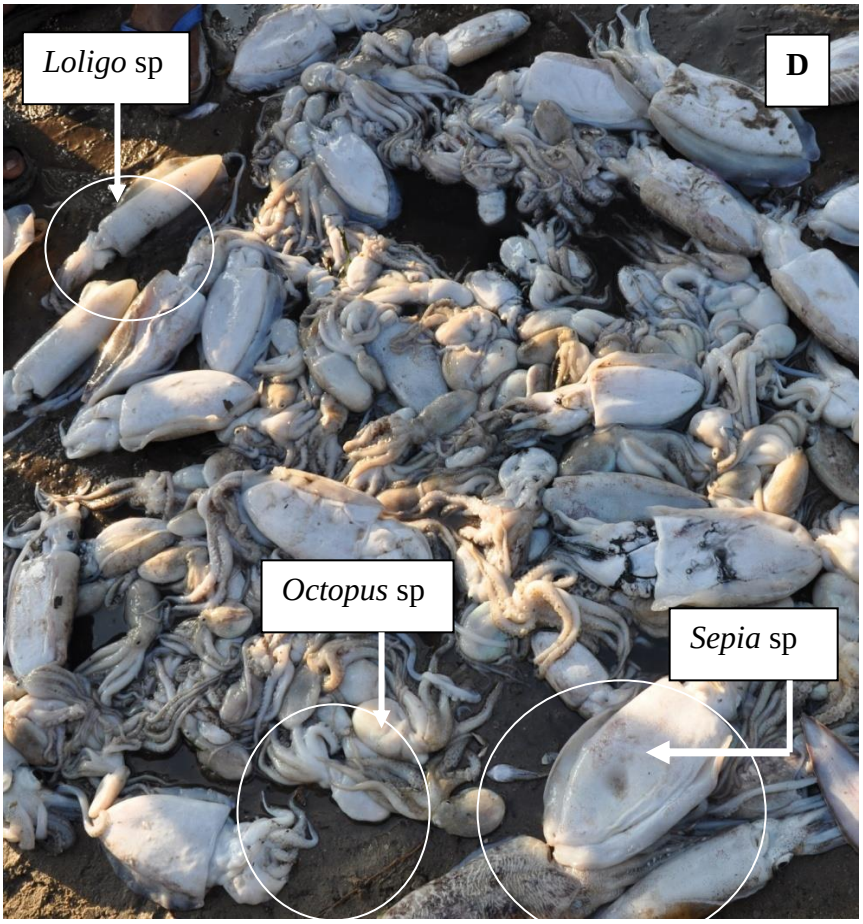


Fig.9.1. Photographs of (**A**) *Loligo duvauceli;* (**B**) *Cistopus indicus,* (**C**) *Octopus membranaceus* and (**D**) Cephalopods at a landing centre of Kerala

Fig.9.2. Collection site of cephalopods in Kerala, India

9.1.3. Preparation of Cephalopod Extracts

The homogenized wet tissues (2 kg each) of the cephalopods, *Loligo duvaucelli*, *Sepia officinalis*, *Octopus membranaceus* and *Cistopus indicus* were extracted with EtOAc: MeOH (1:1, v/v). The extraction process was repeated three times with 2 litres of the extractant and thereafter filtration through a Whatman no. 3 filter paper. The filtrates were concentrated using a rotary evaporator, and the concentrated EtOAc:MeOH extracts were transferred into pre-weighed beakers, dried under N_2 and weighed, before being kept at -20 ºC prior for further processing. The yield of the extract was calculated and expressed as % w/w of the wet tissue.

9.1.4. *In vitro* Anti-Inflammatory Activities of the Cephalopod Extracts

The EtOAc:MeOH extracts were assayed for their *in vitro* anti-inflammatory assays using cyclooxygenase (COX) and Lipoxygenase-5 (LOX-5) enzyme inhibition assays.

9.1.4.1. Cyclooxygenase (COX) Inhibition Assay

Cyclooxygenase (COX-1 and COX-2) inhibition assays were performed using 2, 7-dichlorofluorescein method (Larsen *et al.,* 1996) with suitable modifications. In brief, *leuco*-2, 7-dichlorofluorescein diacetate (5 mg) was hydrolyzed at room temperature in 1 M NaOH (50 µL) for 10 min followed by the addition of 1 M HCl (30 µl) to neutralize the excess of NaOH before the resulting *leuco*-dichlorofluorescein (1-DCF) was diluted in 0.1 M Tris-buffer (pH 8). COX enzyme (COX-1 and COX-2) was diluted in 0.1 M Tris-buffer (pH 8), so that a known aliquot gave an absorbance change of 0.05/min in the test reaction. The test samples (or the equivalent volume of MeOH, 20 µl) were pre-incubated with the enzymes at room temperature for 5 min in the presence of hematin. Premixed phenol, 1-DCF and arachidonic acid were added to the enzyme mixture to begin the reaction, and to give a final reaction mixture of arachidonic acid (50 µM), phenol (500 µM), 1-DCF (20 µM) and hematin (1 µM) in 1 ml final volume of 0.1 M Tris-buffer (pH 8). The reaction was recorded spectrophotometrically over 1 min at 502 nm. A blank reaction mixture (without enzyme) was analyzed in the spectrophotometer reference cell against each test reaction to account for any non-enzymatic activity attributed to the test sample.

9.1.4.2. Lipoxygenase-5 (LOX) Inhibition Assay

The lipoxygenase-5 (LOX-5) inhibition assay was carried out using the principle of 1-4 diene (linoleic acid) oxidation to 1- 3-diene (Baylac & Racine 2003) with modification. Briefly, an aliquot of the stock solution (50 µL, in DMSO and Tween 20 mixture; 29:1, w/w) of each test sample was placed in a 3 ml cuvette, followed by addition of pre-warmed 0.1 M potassium phosphate buffer (2.95 ml, pH 6.3) and linoleic acid solution (48 µl). Thereafter, ice-cold buffer (potassium phosphate) (12 µl) were added with LOX-5 (100 U) before being recorded spectrophotometrically at a wavelength of 234 nm. The control was prepared only with DMSO:Tween 20 mixture (no enzyme inhibition).

9.1.5. Fatty acid composition of *Loligo duvauceli*

The fatty acid composition of *Loligo duvauceli* was performed by the method described in *Chapter 3*.

9.1.6. *In vivo* Anti-inflammatory Activity of the *Loligo duvauceli* Extract

The *in vivo* carrageenan-induced mice paw edema experiment was carried out as previously described (Winter *et al.,* 1962; Chakraborty *et al.,* 2014) with modifications (*Chapter 9*). The animals were divided into 5 groups of 6 animals each. The *Loligo duvauceli* extract mixed in 1% Tween 80 was orally administered at 20 mg/kg. The other groups were injected with either the reference drug (aspirin, 5 mg/kg, orally) or vehicle (Tween 80). After thirty minutes, 1% solution of carrageenan in saline (0.1 ml/mice) was injected subcutaneously into the right hind paw. Paw volumes were measured at 0, 2, 3, 4, 5 and 6 h after injection, and the thickness of the edema was measured with an electronic micrometer (aerospace; 0-25 mm range, least count: 0.001 mm).

9.1.7. Purification of *Loligo duvaucelli*

The EtOAc: MeOH extract (20 g) of *L. duvauceli* was chromatographed over silica column (60-120 mesh) using stepwise gradient of solvents ($CHCl_3$: MeOH) to obtain nine fractions. These fractions were concentrated under vacuum and evaluated for its antioxidant activity by measuring its ability to scavenge DPPH (0.1 mg/ml) and to inhibit the COX-2 and LOX-5 enzymes (0.05 mg/ml). The highest active fractions LD-1, 2, 3 and 9 were further purified using silica column (60-120 mesh)/

preparative normal phase TLC (p-TLC). The schematic diagram is as shown below (Fig. 9.3). The COX-2 and LOX-5 inhibitory activities of the purified fractions (with higher yield) were evaluated (at 0.5 mg/ml concentration)

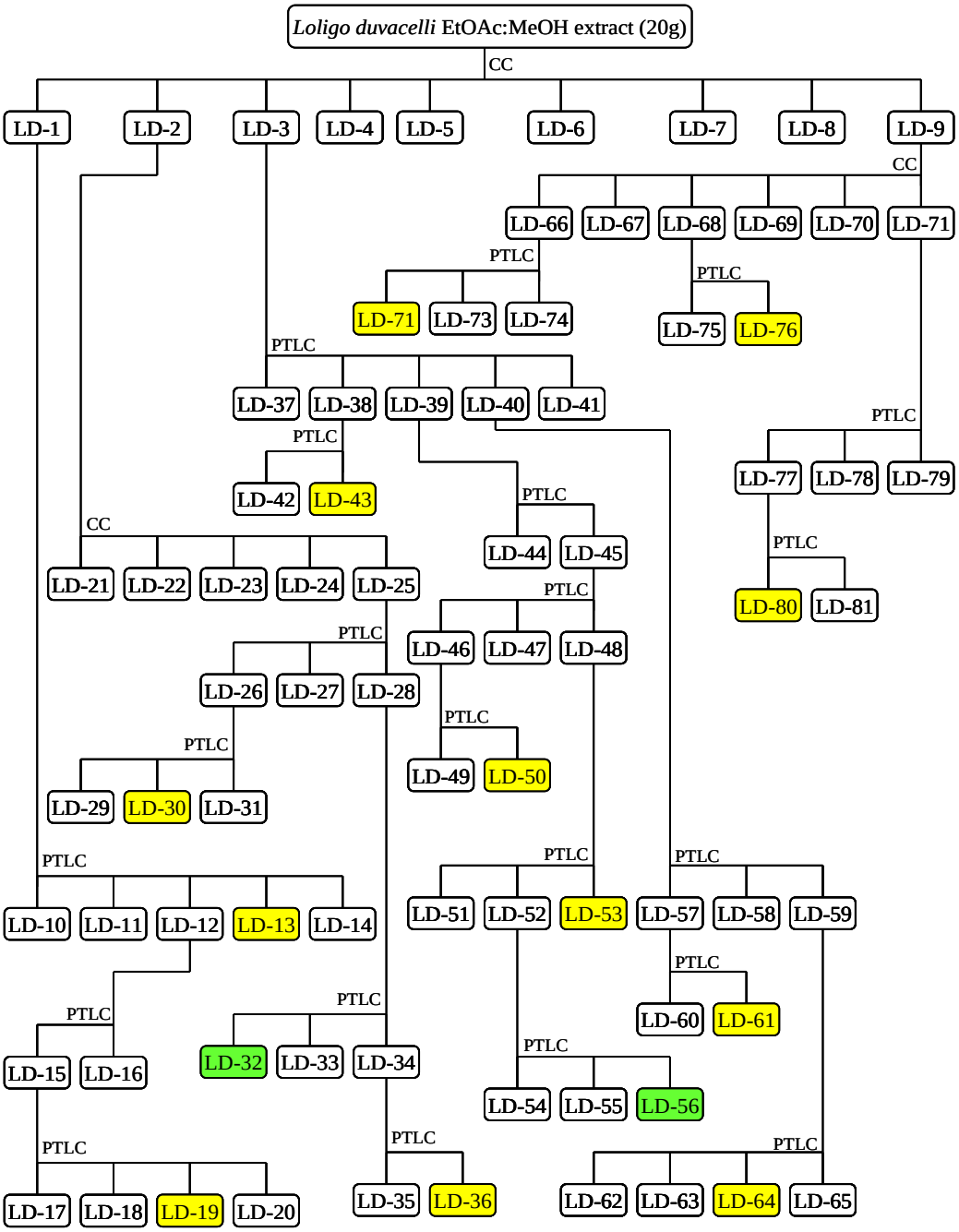


Fig.9.3. Schematic diagram representing the chromatographic purification of the EtOAc:MeOH extract of *Loligo duvauceli* (yellow highlight implies pure compound with higher activity but lower yield < 20mg; green highlight implies pure compound with higher activity & higher yield which considered for structural characterization)

9.1.8. DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of the purified fractions of *Loligo duvaucelli* were carried out following established method (Singh *et al.*, 2002) with suitable modification. Briefly, a solution of the fractions (2 ml, 5 mg/ml) in MeOH were taken in a vial to which added with DPPH (0.1 mM, 2 ml) in distilled water. The reaction mixture was allowed to stand for 30 min at RT, and the absorbance was measured at 517 nm using BHT as reference standard against MeOH as blank in a spectrophotometer. The $DPPH^{\cdot}$ radical scavenging activity was expressed as Total antioxidant radical scavenging activity (% TARSA) = {(absorbance of control-absorbance of sample) × 100}/ absorbance of control.

9.1.9. Other Analyses

The pure compounds were analyzed using Fourier Transform Infra Red spectrometer (FTIR), Mass spectrometry (MS) and Nuclear magnetic resonance spectroscopy (NMR) and the protocols are explained as in the previous chapter (*Chapter 8*).

9.1.10. Statistical Analysis

Statistical evaluation was carried out with the Statistical Program for Social Sciences 13.0 (SPSS Inc, Chicago, USA, ver. 13.0). Analyses were carried out in triplicate, and the means of all parameters were examined for significance by analysis of variance (ANOVA). The level of significance for all analyses was $p \leq 0.05$.

9.2. Results and Discussion

9.2.1. Yield and *In vitro* Anti-Inflammatory Properties of Marine Cephalopod Extracts

The yield of *Loligo duvaucelli* was found to be maximum (4.34 %) followed by *Octopus membranaceus* (2.08 %), *Sepia officinalis* (1.56 %) and *Cistopus indicus* (1.11 %).

Regarding the *in vitro* anti-inflammatory properties, *L. duvaucelli* extract was found to be more effective in inhibiting COX-2 and LOX-5 inflammatory enzymes

than other species (Table 9.1) ($p<0.05$). A dose dependent inhibition of COX-1 and COX-2 enzymes was exhibited by all the cephalopod species studied. The COX-2 inhibition assay used in this study is based on the oxidation of (1-DCF) by the hydroperoxide formed in the COX reaction. *L. duvaucelli* extract at 5 mg/ml showed a comparable inhibition (56.4 %) with aspirin and indomethacin (58.6 & 59 %, respectively; at 5 mg/ml), against pro-inflammatory COX-2 enzymes. Mammalian LOXs have been implicated in the pathogenesis of several inflammatory disorders and are, therefore, important targets for their selective inhibition. LOXs catalyze the dioxygenation of polyunsaturated fatty acids/PUFAs (20:4*n*-6; ARA), which contain one or more 1, 4-*cis*, *cis*-pentadiene units to yield hydroperoxide products. As in COX-2, charge/π-electron and hydrophobic interactions in the catalytic domain of lipoxidase play a major role in catalyzing ARA to yield hydroperoxide products. In this study, a dose-dependent LOX-5 inhibitory activity was exhibited by all the species studied. The inhibition against LOX-5 inflammatory enzymes by *L. duvaucelli* extract (55.4 %) was found to be comparable with the LOX-5 inhibitory activity of aspirin (54.8 %). The lower LOX-5 inhibition by *C. indicus*, *S. officinalis* and *O. membranaceous* was may be due to the absence of potential anti-inflammatory compounds in their tissues.

COX-1 is termed as the housekeeper enzyme due to its presence in most tissues, particularly gastrointestinal tract lining. COX-2 is inducible by the inflammatory response, and increases in response to inflammation including arthritis. It is desirable to inhibit this pro-inflammatory enzyme to deter inflammation. COX-1 maintains normal gastric mucosa, and, therefore, it is undesirable to inhibit this enzyme in an exclusive manner. COX-1/2 ratio apparently provides us with the selectivity of the anti-inflammatory agent/s. Analysis of COX-1/2 ratio and side effects of the NSAIDs show that the higher the ratio, the lower the selectivity towards inhibiting inflammation, and consequently higher are a side effect. Earlier studies reported that (Engelhardt *et al.*, 1995). COX-1/2 ratio of tenoxicam in the order of 15, diclofenac, 2.2, tenidap 122, and piroxicam 33, which are undesirable indicators of an ideal anti-inflammatory agent. In the recent context, selective COX-2 inhibitors are gaining importance as potential new generation selective anti-

inflammatory agents. In the present study, the extracts of *L. duvaucelli*, *S. officinalis* and *C. indicus* realized the COX-1/2 ratios less than 1.0, as compared to the synthetic drugs, aspirin and indomethacin (COX-1/2 > 1.0). A lower COX-1/2 ratio of *L. duvaucelli*, *S. officinalis* and *C. indicus*, especially, *L. duvaucelli* relates to less gastrointestinal side-effect profiles, and effectively manages the manifestations of pain and arthritis (Table 9.1).

In recent times, interest has been focused towards lipoxygenase (LOX) inhibitors, which were reported to block the synthesis of leukotriene metabolites. Though LOX-5, LOX-12, and LOX-15 were known to be involved in leukotriene biosynthesis, LOX-5 appears to be the key enzyme. Accordingly, a lower COX-1/LOX-5 (<1.0) (Table 9.1) indicates higher selectivity and lower side-effect profiles of the cephalopod extracts. Ideally, the simultaneous inhibition of COX-2 and LOX-5 maximize the anti-inflammatory effects, and, therefore, the *L. duvaucelli* extract with lower COX-1/2 and COX-1/LOX-5 shows their higher selectivity against inflammatory response and lower undesirable side effects or toxicity.

Table 9.1 *In vitro* anti-inflammatory activity of different cephalopods compared with standard inflammatory drugs, aspirin and indomethacin.

Concentration (mg/ml)	*Loligo duvaucelli*	*Cistopus indicus*	*Sepia officinalis*	*Octopus membranaceous*	Aspirin	Indomethacin
COX-1 inhibitory activity (%)						
0.5	33.1 ± 0.02^{a}	28.1 ± 0.01^{b}	26.56 ± 0.01^{b}	29.49 ± 0.01^{ab}	44.6 ± 0.02^{c}	45.6 ± 0.02^{d}
1	36.14 ± 0.02^{a}	30.48 ± 0.02^{b}	31.01 ± 0.02^{ab}	41.04 ± 0.02^{c}	52.1 ± 0.03^{d}	54 ± 0.03^{e}
5	42.3 ± 0.02^{a}	36.31 ± 0.02^{b}	37.45 ± 0.02^{b}	50.46 ± 0.03^{c}	70 ± 0.04^{d}	72.1 ± 0.04^{e}
COX-2 inhibitory activity (%)						
0.5	35.26 ± 0.02^{ab}	34.1 ± 0.02^{ab}	32.46 ± 0.02^{a}	32.49 ± 0.02^{a}	38.5 ± 0.02^{b}	34.6 ± 0.02^{ab}
1	50.31 ± 0.03^{a}	41.06 ± 0.02^{b}	37.46 ± 0.02^{c}	39.86 ± 0.02^{c}	52.2 ± 0.03^{a}	46.3 ± 0.02^{ab}
5	56.4 ± 0.03^{a}	47.45 ± 0.02^{b}	46.66 ± 0.02^{b}	50.55 ± 0.03^{b}	58.6 ± 0.03^{a}	59 ± 0.03^{a}
LOX-5 inhibitory activity (%)						
0.5	29.21 ± 0.01^{a}	28.9 ± 0.01^{a}	30.13 ± 0.02^{a}	38.06 ± 0.02^{b}	31.3 ± 0.02^{a}	32.9 ± 0.02^{a}
1	44.62 ± 0.02^{ac}	32.46 ± 0.02^{b}	33.61 ± 0.02^{b}	49.45 ± 0.02^{a}	40.2 ± 0.02^{a}	47.2 ± 0.02^{c}
5	55.36 ± 0.03^{a}	36.31 ± 0.02^{b}	38.45 ± 0.02^{b}	55.14 ± 0.03^{a}	54.8 ± 0.03^{a}	66.3 ± 0.03^{b}
COX-1/COX-2 ratio						
0.5	0.94	0.82	0.82	0.91	1.16	1.32
1	0.72	0.74	0.83	1.03	1	1.17
5	0.75	0.77	0.8	1	1.19	1.22
COX-1/LOX-5 ratio						
0.5	1.13	0.97	0.88	0.77	1.42	1.39
1	0.81	0.94	0.92	0.83	1.3	1.14
5	0.76	1	0.97	0.92	1.28	1.09

*Results are expressed as % activity with respect to the samples, and as mean ± SD (n = 3). a, b, etc: Row wise values with different superscripts of this type indicate significant differences ($p<0.05$) within different samples; COX-1/2 and COX-1/LOX-5 are referred to as selectivity indicators of anti-inflammatory properties of the cephalopod extracts *vis-à-vis* synthetic NSAIDs. A ratio of COX-1/2 and COX-1/LOX-5 more than 1.0 indicates lower anti-inflammatory selectivity and adverse reactions towards the mammalian gastrointestinal tract

9.2.2. *In vivo* Anti-inflammatory Properties of *Loligo duvaucelli*

L. duvaucelli extract showed maximum anti-inflammatory potential against the *in vitro* enzymes. Hence, they were further evaluated for their activities in an *in vivo* system. The effect of the *L. duvaucelli* extract compared with the standard drug (aspirin) on carrageenan-induced hind paw edema in BALB/C mice is recorded in Table 9.2. The subplantar injection of carrageenan in control mice induced an increase in paw thickness over 6 h. In the carrageenan administered animals severe swelling was observed at 5^{th} h, and the swelling was maintained until 6^{th} h. The aspirin treated groups decreased paw edema significantly ($p<0.05$) with a maximum inhibition of 82.6 % after 6^{th} h. Macrides & Kalafatis (2000) compared the effects of aspirin, ibuprofen and stabilized freeze-dried *P. canaliculus* extract in carageenan-induced inflammation in rodents, which revealed that aspirin reduced inflammatory swelling by 40 %, ibuprofen by 60 % and stabilized mussel extract by 90 %. Notably, the animals challenged with *L. duvaucelli* extract significantly mitigated the carrageenan-induced inflammation (edema/swelling) in rats in a time-dependent manner till the end of the 6^{th} h as compared to the carrageenan administered animals throughout the period of study ($p<0.05$). *L. duvaucelli* extract showed inhibition of the edema to the tune of 46.4 % (20 mg/kg dose) during the 3^{rd} h after the carrageenan injection, which reached to 90.7 %, respectively after the 6^{th} h of treatment as compared to 82.6 % for standard synthetic drug (aspirin). However, it was interesting to note that the activity of *L. duvaucelli* extract persisted throughout the period of study, the activities improved with time, indicating their potential to release the active principle throughout an extended period.

Table 9.2 Effect of *Loligo duvaucelli* extract (20 mg/kg) compared with the standard drug (aspirin 5mg/kg) on carrageenann-induced hind paw edema in BALB/C mice

Samples	Paw edema (mm)	Paw edema (mm)	Difference in paw edema (%)*	(% inhibition)	Paw edema (mm)	Difference in paw edema (%)*	(% inhibition)	Paw edema (mm)	Difference in paw edema (%)*	(% inhibition)	Paw edema (mm)	Difference in paw edema (%)*	(% inhibition)	Paw edema (mm)	Difference in paw edema (%)*	(% inhibition)
	0h	2h	2h	2h	3h	3h	3h	4h	4h	4h	5h	5h	4h	6h	6h	6h
Normal saline	1.16	2.49	114.66		2.51	116.38		2.52	117.24		2.53	118.1		2.54	118.97	
Standard	1.46	2.22	52.05	54.6	2.05	40.41	65.28	1.99	36.3	69.04	1.92	31.51	73.32	1.88	28.77	75.82
Loligo duvaucelli	1.46	2.35	60.96	46.83	2.11	44.52	61.75	2.05	40.41	65.53	2.02	38.36	67.52	2.03	39.04	67.18

*Difference in mice paw edema with respect to zeroth hour.

Percent difference in mice paw edema was calculated as: $(T_t - T_o)$ X 100/ T_o, where T_t is the average thickness of the edema for each group after treatment in different time intervals and T_o is the average thickness for each group at baseline.

Percent inhibition in mice paw edema (in parentheses) was calculated as: $(I_t - I_o)$ X 100/ I_o, where I_t is the percent difference in paw edema of the animals treated with normal saline with respect to the baseline value (zeroth hour) and I_o is the percent difference in paw edema of animals treated with samples/standard at the same time interval.

9.2.3. Fatty Acid Composition of *Loligo duvaucelli*

The results of the fatty acid composition analysis show that cephalopods are rich in *n*-3 fatty acids. It is interesting to note that the C22 polyunsaturated fatty acid docosahexaenoic acid (22:6*n*-3) was found to be higher than C20 fatty acid EPA (20:5*n*-3). Among the saturated fatty acids 16:0 was found to be predominant. The greater share of polyunsaturated fatty acids of *n*-3 class than the saturated fatty acids signify the beneficial effect of the loligo fatty acids. These results are in agreement with previous studies on fatty acids of cephalopods (Ozyurt *et al.,* 2006; Reale *et al.,* 2006). For cephalopods the major polyunsaturated fatty acids are C20:5, n-3 and C22:6, *n*-3 acids (de Koning 1993; Forneris *et al.,* 1981; Joseph 1982). *Loligo vulgaris* has been found to contain 2.5% of lipids of the mantle (edible part) (de Koning 1993). Adult cephalopods are rich in long chain polyunsaturated fatty acids PUFA (Ackman *et al.,* 1995; Nash *et al.,* 1978), their oil, being a good source of these compounds, often used in aquaculture to supplement feeds (Southgate & Lou 1995; Knauer & Southgate 1997). The GC profile is shown in Fig. 9.4.

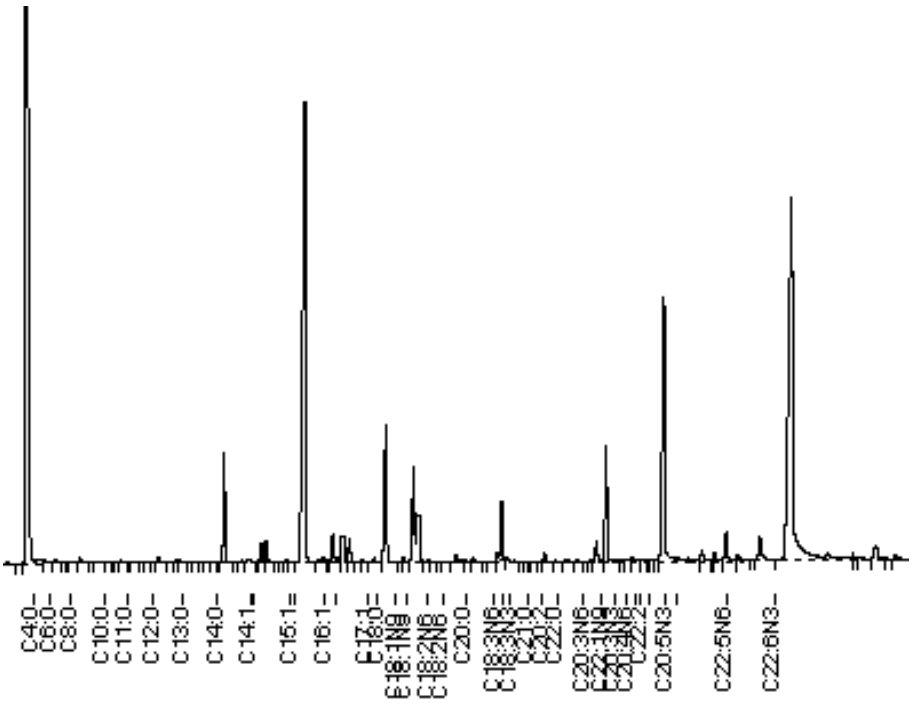


Fig.9.4. GC chromatogram of *Loligo duvaucelli* wet tissue

9.2.4. Purification of *Loligo duvaucelli*

The nine fractions obtained from the EtOAc: MeOH extract of *L. duvaucelli* were studied for their *in vitro* anti-inflammatory properties, and the fractions LD-1, LD-2, LD-3 and LD-9 were found to possess >40% DPPH radical scavenging activity with >20% COX-2 inhibitory activity and >10% LOX-5 inhibitory activity (Table 9.3).

The fraction LD-1 (1230 mg) was further purified over p-TLC using 10%EtOAc:*n*-hexane to obtain five fractions of which LD-13 (low yield) was found to be pure with 31.24 & 18.5 % COX-2 and LOX-5 inhibitory activities, respectively, and 70.1 % DPPH radical scavenging activity. However, LD-12 which showed similar DPPH radical scavenging activity (70.1 %) on further purification yielded LD-19 as pure compound but with low yield (10 mg).

Likewise, the fraction LD-2 on sequential purification through p-TLC yielded LD-32 and LD-36 as pure compounds with 68.6 & 72.4 % DPPH radical scavenging activity. Among these, LD-32 possessed higher yield (65 mg, COMPOUND I) but, LD-36 with lower yield (≤ 10 mg). LD-32 showed high activity in inhibiting COX-2 and LOX-5 enzymes along with high radical scavenging activity.

The fraction LD-3 on purification using p-TLC (10% EtOAc:*n*-hexane) yielded LD-37 to LD-41. LD-39 with higher DPPH radical scavenging activity (60.2 %) was further chromatographed by sequential p-TLC using 10% EtOAc:*n*-hexane, 30% EtOAc:*n*-hexane to get LD-56 as pure compound (COMPOUND II, 55 mg) with > 25 % COX-2 and LOX-5 inhibitory activities and 71.7 % DPPH radical scavenging activity. Similarly, LD-40 on chromatography using p-TLC yielded LD-61 and LD-64 as pure, active compounds but a low yield was observed for these compounds (≤ 11 mg) (Table 9.3).

LD-9 on column purification yielded six fractions LD-66 to LD-71. However, LD-68 and LD-71 among these fractions yielded two pure compounds, LD-76 and LD-80 with high DPPH radical scavenging activity (> 70 %) these compounds were less than 7 mg.

Table 9.3 Yield, Rf, Radical scavenging and *in vitro* anti-inflammatory activities of chromatographic purification of *Loligo duvaucelli* extract

	Yield (mg)	Rf	DPPH radical	COX-2	LOX-5
LD CC (M/C)					
LD-1(100% C)	1230		68.66	30.1	20.16
LD-2(5% M/C)	1560		50.36	26.21	13.11
LD-3(10% M/C)	2550		47.06	28.16	21.14
LD-4(15% M/C)	2980		0.36	0.24	0
LD-5(30% M/C)	1020		4.87	0	0
LD-6(40% M/C)	2010		10.89	0.17	0.08
LD-7(50% M/C)	3140		20.06	10.46	0
LD-8(80% M/C)	2010		0.84	0	7.65
LD-9(90% M/C)	1220		56.98	26.49	17.64
LD-1 (PTLC 10% E/H)					
LD-10	80	0.24	0.7	NA	NA
LD-11	50	0.51	0.74	NA	NA
LD-12	740	0.66	70.06	NA	NA
LD-13	10	0.78	70.06	31.24	18.46
LD-14	290	0.92	1.44	NA	NA
LD-12(PTLC 10% E/H)					
LD-15	250	0.25	52.46	NA	NA
LD-16	430	0.62	0.87	NA	NA
LD-15(PTLC 20% E/H)					
LD-17	20	0.16	11.51	NA	NA
LD-18	55	0.48	10.64	NA	NA
LD-19	10	0.56	60.46	NA	NA
LD-20	150	0.78	0.87	NA	NA
LD-2(PTLC 10% E/H)					
LD-21	230	0.06	9.44	NA	NA
LD-22	600	0.11	12.86	NA	NA
LD-23	80	0.24	15.06	NA	NA
LD-24	101	0.46	0.87	NA	NA
LD-25	530	0.89	50.49	NA	NA
LD-25(PTLC 1% M/C)					
LD-26	200	0.14	60.44	NA	NA
LD-27	210	0.59	1.87	NA	NA
LD-28	110	0.77	59.56	NA	NA
LD-26(PTLC 1% M/C)					
LD-29	58	0.16	14.01	NA	NA
LD-30	75	0.49	60.14	NA	NA
LD-31	55	0.88	0.06	NA	NA
LD-28(PTLC 1% M/C)					
LD-32 COMPOUND I	65	0.8	68.64	37.4	33.01
LD-33	10	0.49	0.01	NA	NA

LD-34	28	0.81	50	NA	NA
LD-34(PTLC 1% M/C)					
LD-35	18	0.16	0.84	NA	NA
LD-36	8	0.54	72.43	NA	NA
LD-3(PTLC 10% E/H)					
LD-37	60	0.24	1.26	NA	NA
LD-38	512	0.51	41.84	NA	NA
LD-39	480	0.62	60.16	NA	NA
LD-40	526	0.86	66.21	NA	NA
LD-41	840	0.94	0.88	NA	NA
LD-38					
LD-42	200	0.16	15.78	NA	NA
LD-43	300	0.27	55.87	34.11	21.89
LD-39(PTLC 10% E/H)					
LD-44	265	0.34	0.14	NA	NA
LD-45	208	0.78	43.8	NA	NA
LD-45(PTLC 10% E/H)					
LD-46	30	0.14	59.44	NA	NA
LD-47	60	0.52	0.11	NA	NA
LD-48	105	0.87	61.51	NA	NA
LD-46(PTLC 1% M/C)					
LD-49	65	0.16	NA	NA	NA
LD-50	37	0.66	67.62	40	31.32
LD-48(PTLC 30% E/H)					
LD-51	0.5	0.21	0.64	NA	NA
LD-52	100	0.48	48.45	NA	NA
LD-53	0.2	0.77	70.16	36.24	30.11
LD-52(PTLC 30% E/H)					
LD-54	5.1	0.11	45.64	NA	NA
LD-55	0.2	0.42	NA	NA	NA
LD-56 COMPOUND II	84	0.82	71.68	36.21	30.13
LD-40(PTLC 40% E/H)					
LD-57	212	0.41	46.41	NA	NA
LD-58	160	0.81	0.41	NA	NA
LD-59	135	0.95	50.12	NA	NA
LD-57(PTLC 30% E/H)					
LD-60	200	0.24	13.24	NA	NA
LD-61	5	0.89	52.61	37.24	2.1
LD-59(PTLC 70% E/H)					
LD-62	35	0.29	0	NA	NA
LD-63	22	0.67	22.16	NA	NA
LD-64	11	4.86	70.24	18.22	16.31
LD-65	55	7.88	14.46	NA	NA
LD-9 (CC C/M)					

LD-66(100%C)	280		49.64	NA	NA
LD-67(1%M/C)	102		10.12	NA	NA
LD-68(10%M/C)	225		50.24	NA	NA
LD-69(15%M/C)	98		4.68	NA	NA
LD-70(20%M/C)	120		6.12	NA	NA
LD-71(50%M/C)	385		66.16	NA	NA
LD-66(PTLC 5% M/D)					
LD-72	105	0.36	80	22.41	1.46
LD-73	95	0.58	16.45	NA	NA
LD-74	75	0.81	67.69	NA	NA
LD-68(PTLC 5% M/D)					
LD-75	208	0.24	0	NA	NA
LD-76	6.2	0.86	70.16	29.41	3.16
LD-71(PTLC 5% M/D)					
LD-77	185	0.14	67.4	NA	NA
LD-78	98	0.56	0.1	NA	NA
LD-79	100	0.91	22.2	NA	NA
LD-77(PTLC 5% M/D)					
LD-80	3.2	0.26	74.1	29.46	4.87
LD-81	178	0.72	6.2	NA	NA

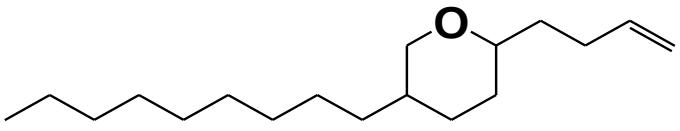
DPPH radical scavenging activity at 0.1 mg/ml is expressed in % (48 h); COX-2 inhibitory activity at 0.1 mg/ml is expressed in %; LOX-5 inhibitory activity at 0.05 mg/ml is expressed in %; NA – NOT ASSAYED ie. the fractions with low yield were evaluated only for COX-2 and LOX-5 inhibitory activity. CC – Column chromatography; PTLC – Preparative thin layer chromatography; M – Methanol; E –Ethyl acetate; H – *n*-hexane; C – Chloroform; D – Dichloromethane; LD - *Loligo duvaucelli*

The results of the detailed chemical investigation of the pure compounds LD - 32 and LD -56 isolated from *Loligo duvaucelli* are as follows (*Section 9.2.4.1 & 9.2.4.2*).

9.2.4.1. Structural Characterization of Compound I (LD-32)

Compound I (LD-32)

2-(But-3-enyl)-tetrahydro-5-nonyl-2H-pyran



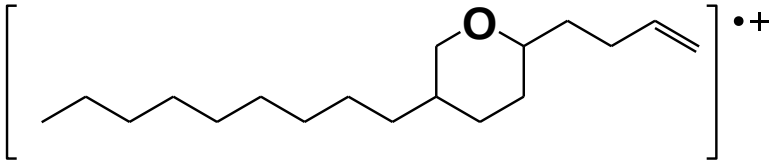
Yield	65 mg (0.33 %*)
Physical description	Pale yellow powder
Molecular formula	$C_{18}H_{34}O$
Molecular weight	266.4752

* % yield based on starting *Loligo duvaucelli* extract

The spectroscopic characterization of the pure and active compound (LD-32), 5-(7-butoxydodecyl)-2-(2-ethyl-3-butenyl)-3, 6-dihydro-2*H*-pyran are discussed in detail below.

2-(But-3-enyl)-tetrahydro-5-nonyl-2H-pyran: Pale yellow powder; UV (MeOH) λ_{max} (log ε): 230 nm (2.61); TLC (Si gel GF_{254} 15 mm; 1% MeOH/$CHCl_3$, v/ v) R_f: 0.8; GC (Elite – 5 capillary column 30 m x 0.53 mm i.d.; oven temperature ramp: 60^{o}C for 10 min, rising at 5^{o}C /min to 220^{o}C; 1 mL injection volume/$CHCl_3$) R_t: 5.20; Elemental analysis found: C,81.13; H,12.86;O,6.01; IR (KBr, cm^{-1}) ν_{max} 722.29 cm^{-1} (C-H rocking), 836.17 cm^{-1} (C-H rocking), 1454.38 cm^{-1} (C-C stretching in ring), 1466.91 cm^{-1} (C-H bending of alkanes), 1642 (C=C stretching), 2923.22 cm^{-1} (C-H stretching of alkanes), 2955.04 cm^{-1} (C-H stretching of alkanes); ^{1}H NMR (500 MHz, Chloroform-d) δ 5.88 – 5.73 (m, 1H), 3.71 – 3.60 (m, 1H), 2.40 (m, 2H), 2.13 – 1.94 (m, 2H), 1.57(m, 2H), 0.88(t, 3H); ^{13}C-NMR, ^{1}H-^{1}H-COSY and HMBC data (Table 9.4); HRESIMS m/e*:* ($C_{18}H_{34}O$): 266.4752 (calcd. for $C_{18}H_{34}O$ 266.4814).

The spectroscopic characterization of the pure and active compound (LD-32), 5-(7-butoxydodecyl)-2-(2-ethyl-3-butenyl)-3, 6-dihydro-2*H*-pyran are discussed in detail as follows. The compound 2-(But-3-enyl)-tetrahydro-5-nonyl-2H-pyran, a new pyran derivative was isolated as pale yellow powder upon repeated chromatography over silica columns/TLC. The ultraviolet absorbances at λ_{max} (log e) 230 (2.61) nm has been assigned to a chromophore with olefinic system. Its mass spectrum exhibited a molecular ion peak at m/e 266 (HRESIMS m/e 289.4628 $[M+Na]^+$; D 0.0 amu, Fig. 9.5), which in combination with its ^{1}H and ^{13}C NMR data (Table 9.4) indicated the elemental composition of $C_{18}H_{34}O$ as 2-(but-3-enyl)-tetrahydro-5-nonyl-2H-pyran with two degrees of unsaturation. One degree of unsaturation from the double bond as assigned by ^{1}H NMR (δ 5.84 and δ 4.98 ppm) in conjugation with ^{13}C-NMR (δ 139.27 and δ 114.05 ppm). The other degree of unsaturation was due to a cyclic ring system, which is not of aromatic type. This is based on the absence of the aromatic proton signal in the ^{1}H NMR spectrum.

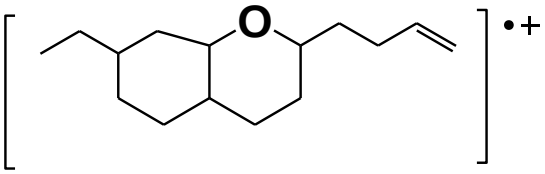


2-(but-3-enyl)-tetrahydro-5-nonyl-2*H*-pyran

$C_{18}H_{34}O^{\bullet+}$

m/e: 266.46

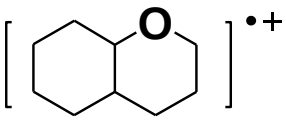
Intramolecular rearrangement



2-(but-3-enyl)-7-ethyl-octahydro-2*H*-chromene

$C_{15}H_{26}O^{\bullet+}$

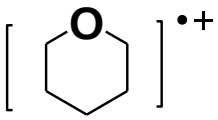
m/e: 222.37



octahydro-2*H*-chromene

$C_9H_{16}O^{\bullet+}$

m/e: 140.22



tetrahydro-2*H*-pyran

$C_5H_{10}O^{\bullet+}$

m/e: 86.13

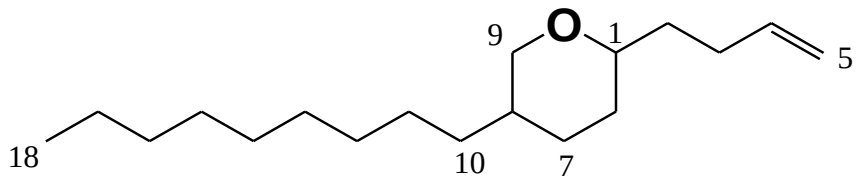
Fig. 9.5. Mass fragmentation pattern of 2-(but-3-enyl)-tetrahydro-5-nonyl-2H-pyran

The ^{1}H NMR in conjugation with ^{13}C-NMR recorded the presence of thirteen methylene carbons and two methine carbons (δ 71.72 and 31.60 ppm). Olefinic signals are apparent at δ 139.27 and 114.05 ppm, indicating the presence of a double bond at the terminal position of the compound. ^{1}H–^{1}H COSY correlations between δ 0.88 (assigned to be as H-18)/ δ 0.91 (H-17)/ δ 1.48 (H-16)/ δ 1.57 (H-15)/ δ 1.31 (H-14)/ δ 1.78 (H-13)/ δ 1.84 (H-12)/ δ 2.08 (H-11)/ δ 2.40 (H-10)/ δ 3.02 (H-10) supports the presence of the nonane moiety in the compound. The proton and carbon connectivity deduced from HSQC and HMBC experiments confirmed the side chain framework (Fig. 9.6.6 & 9.6.7). The relatively downfield shift of the methine proton at δ 3.02 ppm referred to a possible oxygenation in its vicinity. The methine proton at δ 3.02 ppm is characteristic of the junction point of the nonane moiety with that of an oxygenated hydrocarbon network as established by ^{1}H–^{1}H COSY correlations and detailed HMBC experiments (Table 9.4). The presence of two –CH signals at δ 3.02 ppm and δ 3.66 ppm apparently indicate the presence of a cyclic oxygenated ring system in the compound. ^{1}H–^{1}H COSY correlations between δ 3.02 (assigned to be as H-8)/ δ 2.56 (H-9)/ δ 1.67 (H-7)/ δ 2.28 (H-6)/ δ 3.66 (H-1) established the presence of tetrahydro-2H-pyran ring system in the compound. The significant downfield shift of the methine proton at δ 3.66 ppm bearing the carbon atom at δ 71.72 ppm indicate its presence at a junction point attached to a side chain. ^{1}H–^{1}H COSY correlations between δ 1.36 (assigned to be as H-2)/ δ 2.04 (H-3)/ δ 5.84 (H-4) indicate the presence of a C3 fragment attached to the –CH group at δ 3.66 ppm. The presence of the olefinic bond at δ 5.84 and δ 4.98 ppm bearing the carbon atom at δ 139.27 and δ 114.05 ppm has been established by detailed HMBC and HSQC experiments. The protons of the $–CH_2-$ group at δ 2.04 are deshielded due to the presence of the olefinic moiety (-C=C-) at the α-position with respect to the deshielded methylene group. The ^{13}C NMR spectrum of the purified compound in combination with DEPT experiments indicated the occurrence of 18 carbon atoms in the molecule including two methine carbons between δ 33.60 and 57.45 (Table 9.4).

The point of cyclization of the pyran was indicated by the low-field shift of H-1 at δ 3.66, which has been coupled to the H-2 methylene group at δ 1.36, which also gives clear $^{1}H–^{1}H$ COSY correlation (Table 9.4) (Fig. 9.6.4). The proton and carbon connectivity deduced from HSQC and HMBC experiments confirmed the tetrahydro-2H-pyran framework attached to the side chain C-4 moiety at the 1-C position to afford the 2-(but-3-enyl)-tetrahydro-2H-pyran framework. The H–H and C–H connectivities apparent in the $^{1}H–^{1}H$ COSY and HMBC spectra respectively indicate that one of the two unsaturations was due to the pyran ring framework. In the HMBC spectrum, it was observed that H-3 (δ 2.04)/C-4 (δ 139.27), C-5 (δ 114.05), C-2 (δ 31.93), which established the presence of the C4 butene network. Furthermore, the H-C connectivity as illustrated by the HMBC spectrum showed strong correlations between H-8 (δ 3.02)/C-7 (δ 31.44), C-9 (57.45); H-9 (δ 2.56)/C-7 (δ 31.44), C-1 (δ 3.66); and H-9 (δ 2.56)/C-2 (δ 31.93) were correlated with each other (Table 9.4). These experiments further corroborated the presence of the 2-(but-3-enyl)-tetrahydro-2H-pyran framework in the compound. The geometric isomerisms of the olefinic protons have been established by the 1H coupling constants suitable for the geometries (E form). The relative stereochemistry of the chiral centres, particularly that of C-1 (δ 3.66) and C-8 (δ 3.02), was deduced from the NOESY spectrum of the compound and the J-values. The methine proton at C-1 group did not exhibit NOE interactions with H-8, thereby indicating that these groups were disposed at the opposite direction of the plane of reference. The olefinic (C=C), and ether (C-O-C) groups have been symbolized by the absorption bands at 1642 and 1068 cm^{-1} respectively. The characteristic IR absorption spectra at 1454.38 cm^{-1} (C-C stretching in ring), 1466.91 cm^{-1} (C-H bending of alkanes), 1642 (C=C stretching), 2923.22 cm^{-1} (C-H stretching of alkanes), 2955.04 cm^{-1} (C-H stretching of alkanes) also support the structure of 2-(but-3-enyl)-tetrahydro-5-nonyl-2H-pyran. The pseudomolecular ion peak at m/e 289.4628 appeared to undergo intramolecular rearrangement to yield 2-(but-3-enyl)-7-ethyl-octahydro-2H-chromene at m/e 222.37 ($C_{15}H_{26}O^{\bullet+}$), which

undergoes elimination of the side chains (pentenyl and ethyl) to afford a fragment with m/e 140.22 ($C_9H_{16}O^{\bullet+}$) assigned to be as octahydro-2H-chromene. Appearance of the fragment at m/e 86.13 indicates the presence of tetrahydro-2H-pyran moiety ($C_5H_{10}O^{\bullet+}$), resulted from the elimination of cyclohexane fragment.

Tale 9.4 NMR spectroscopic data of 2-(but-3-enyl)-tetrahydro-5-nonyl-2H-pyran in $CDCl_3$.[a]



Carbon no.	^{13}C NMR (DEPT)	H	δ^{1}H NMR (int., mult., J in Hz)[b]	^{1}H-^{1}H COSY	HMBC (^{1}H-^{13}C)
1	71.72	1H	3.71-3.60(m,1H)	H-2	
2	31.93	2H	1.36(m,2H)	H-3	C-3,1
3	33.82	3H	2.13-1.94(m,2H)	H-4	C-4,5
4	139.27	4H	5.88-5.73(m,1H)	H^{b}-5	C-2
5	114.05	5H^{a}	5.01	H-4	C-4
		5H^{b}	4.98		
6	32.76	6H	2.28(m,2H)	H-7	
7	31.44	7H	1.67(m,2H)	H-6	
8	33.60	8H	3.02(m,1H)	H-9,10	C-7,9
9	57.45	9H	2.56(d,2H)	H-8	C-1
10	29.66	10H	2.40(m,2H)	H-11,8	
11	29.70	11H	2.08(m,2H)	H-12	C-12
12	29.62	12H	1.84(m,2H)	H-13	
13	29.36	13H	1.78(m,2H)		C-10
14	29.36	14H	1.31(m,2H)		
15	29.16	15H	1.57(m,2H)	H-16	C-16
16	22.75	16H	1.48(m,2H)	H-17	C-18
17	19.75	17H	0.91(m,2H)	H-18	C-15
18	14.10	18H	0.88(t,3H)		C-15,17

[a] NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers.
[b]Values in ppm, multiplicity and coupling constants (J¼ Hz) are indicated in parentheses. Assignments were made with the aid of the ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY experiments.

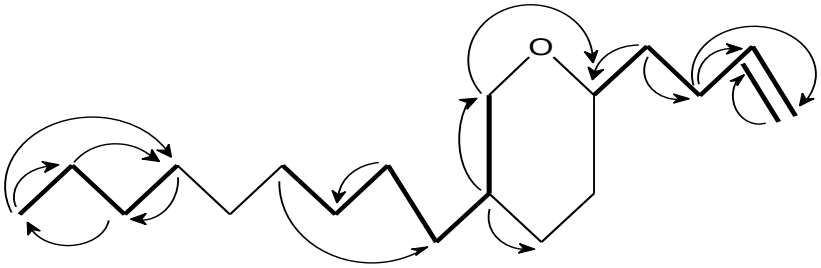


Fig. 9.6.1. ^{1}H—^{1}H COSY and HMBC correlations of 2-(but-3-enyl)-tetrahydro-5-nonyl-2H-pyran. The key 1H-1H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

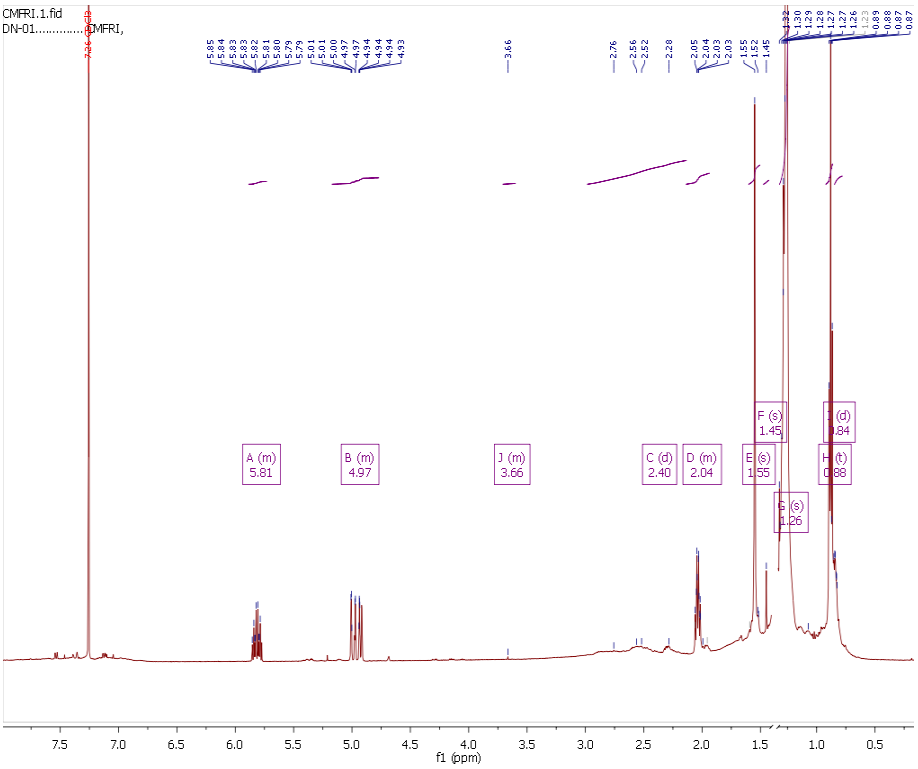


Fig. 9.6.2. ^{1}HNMR spectrum of 2-(but-3-enyl)-tetrahydro-5-nonyl-2H-pyran

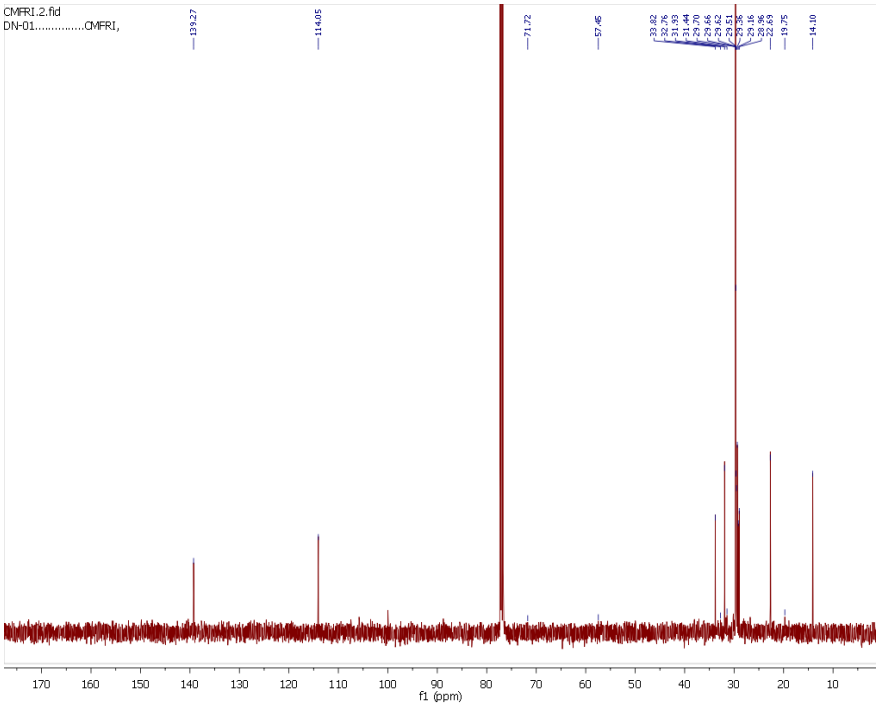


Fig. 9.6.3. ^{13}C NMR spectrum of 2-(but-3-enyl)-tetrahydro-5-nonyl-2H-pyran

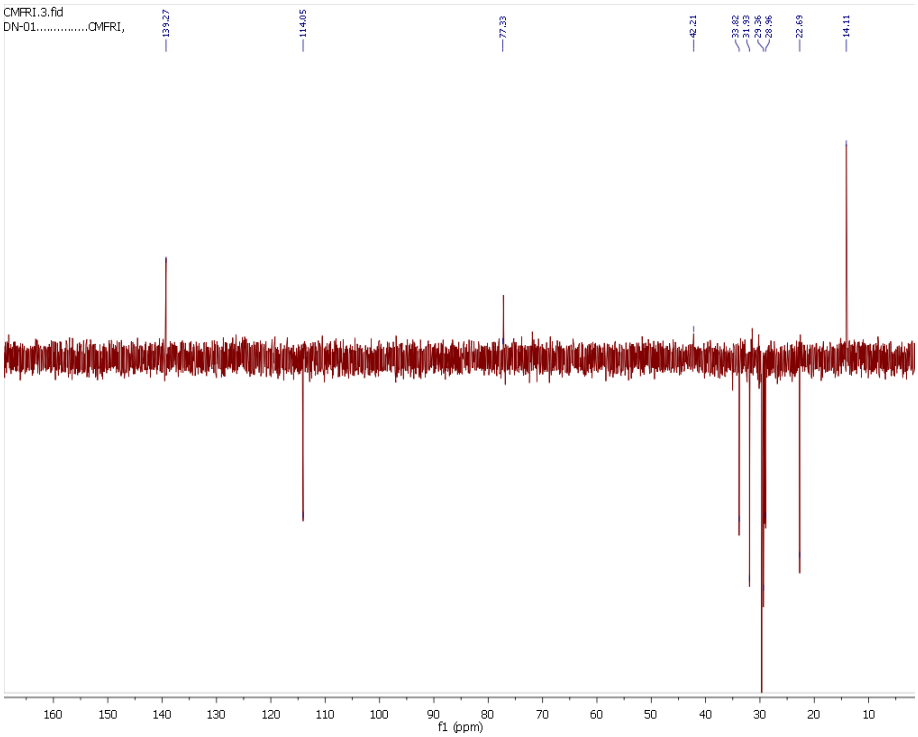


Fig. 9.6.4. DEPT spectrum of 2-(but-3-enyl)-tetrahydro-5-nonyl-2H-pyran

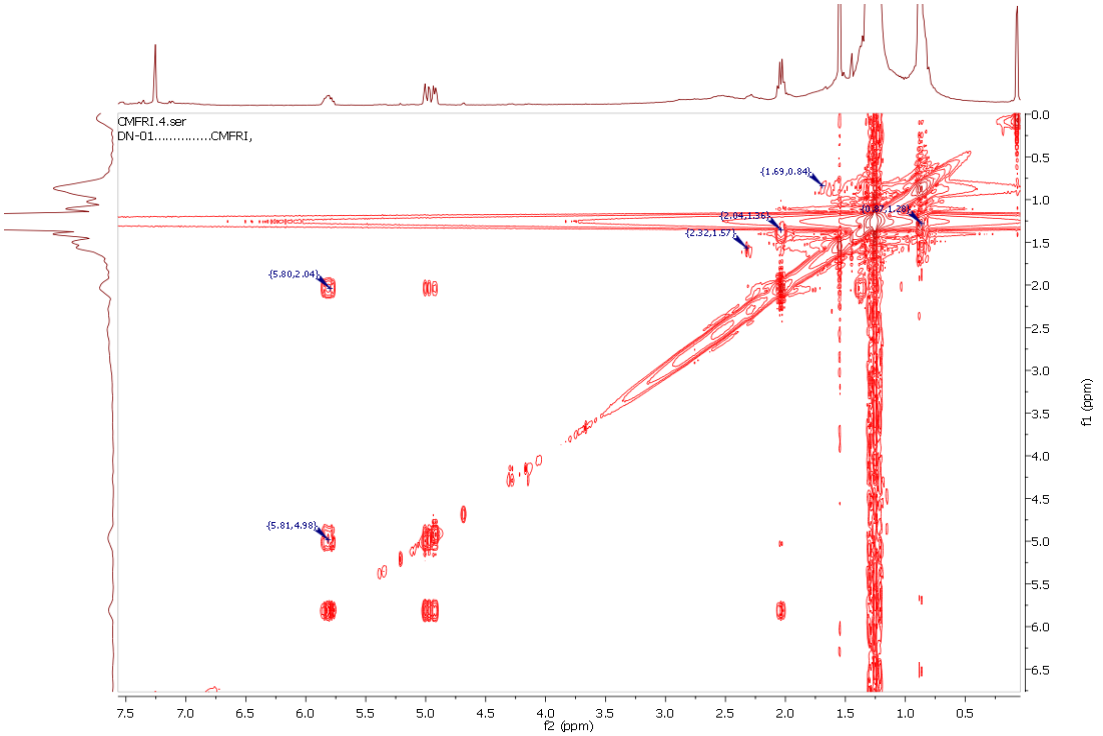


Fig. 9.6.5. ^{1}H—^{1}H COSY spectrum of 2-(but-3-enyl)-tetrahydro-5-nonyl-2H-pyran

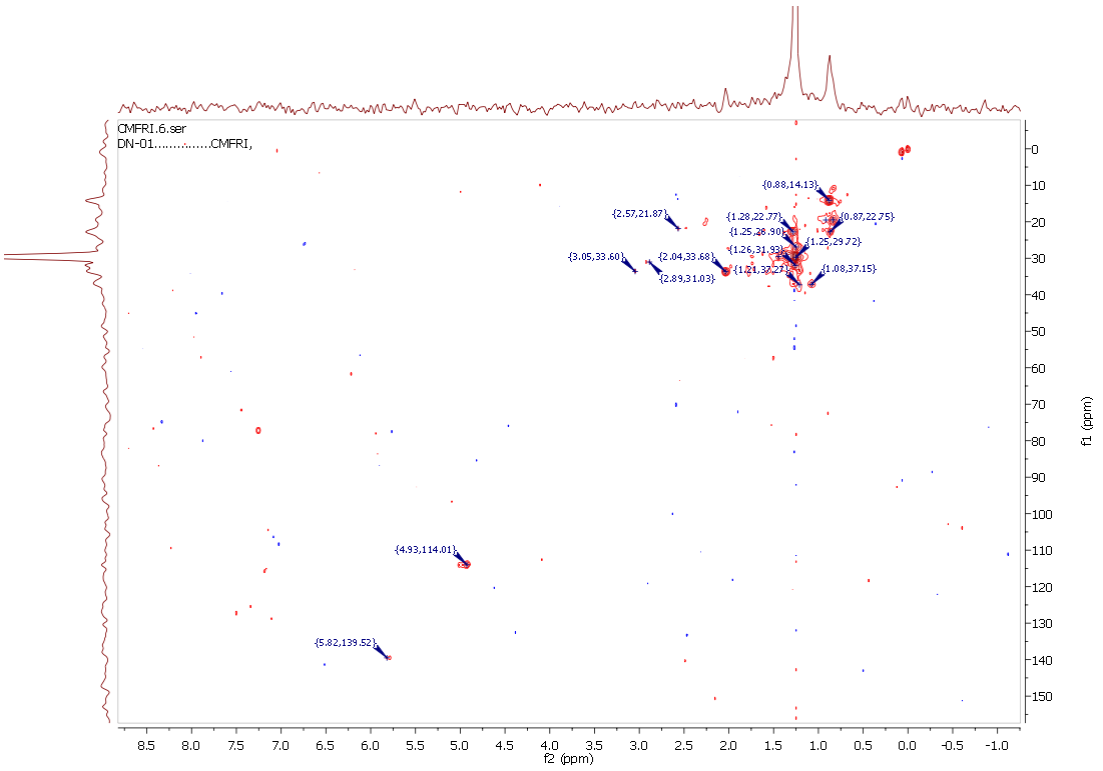


Fig. 9.6.6. HSQC spectrum of 2-(but-3-enyl)-tetrahydro-5-nonyl-2H-pyran

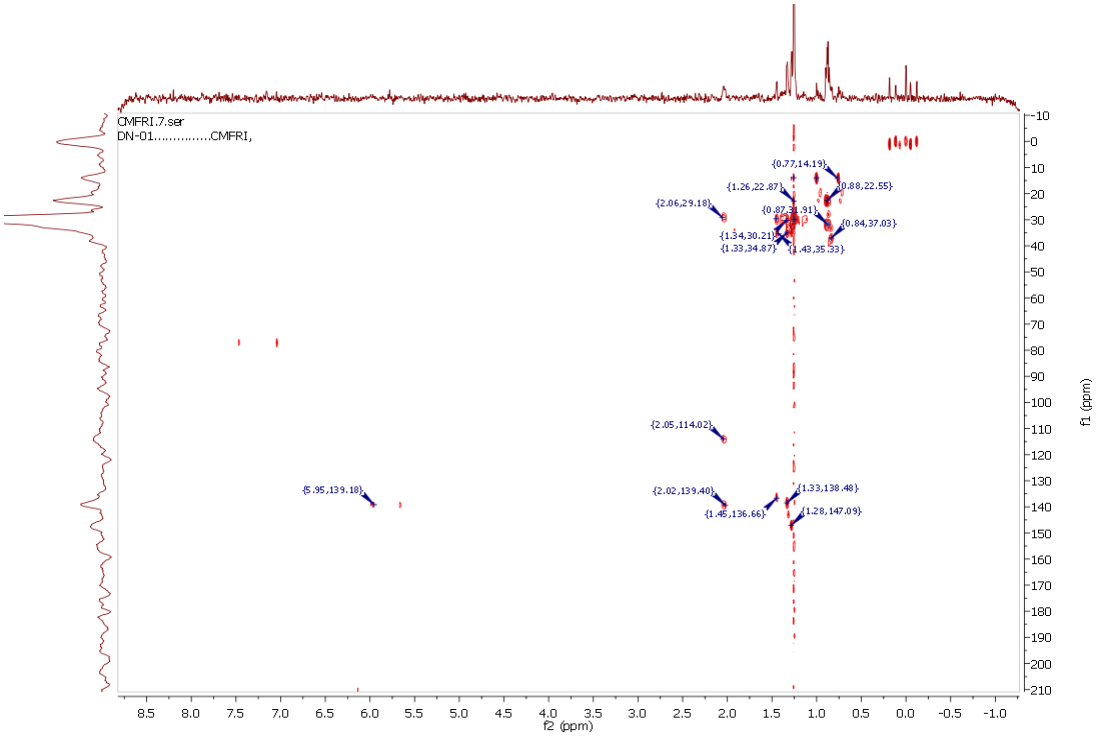


Fig. 9.6.7. HMBC spectrum of 2-(but-3-enyl)-tetrahydro-5-nonyl-2H-pyran

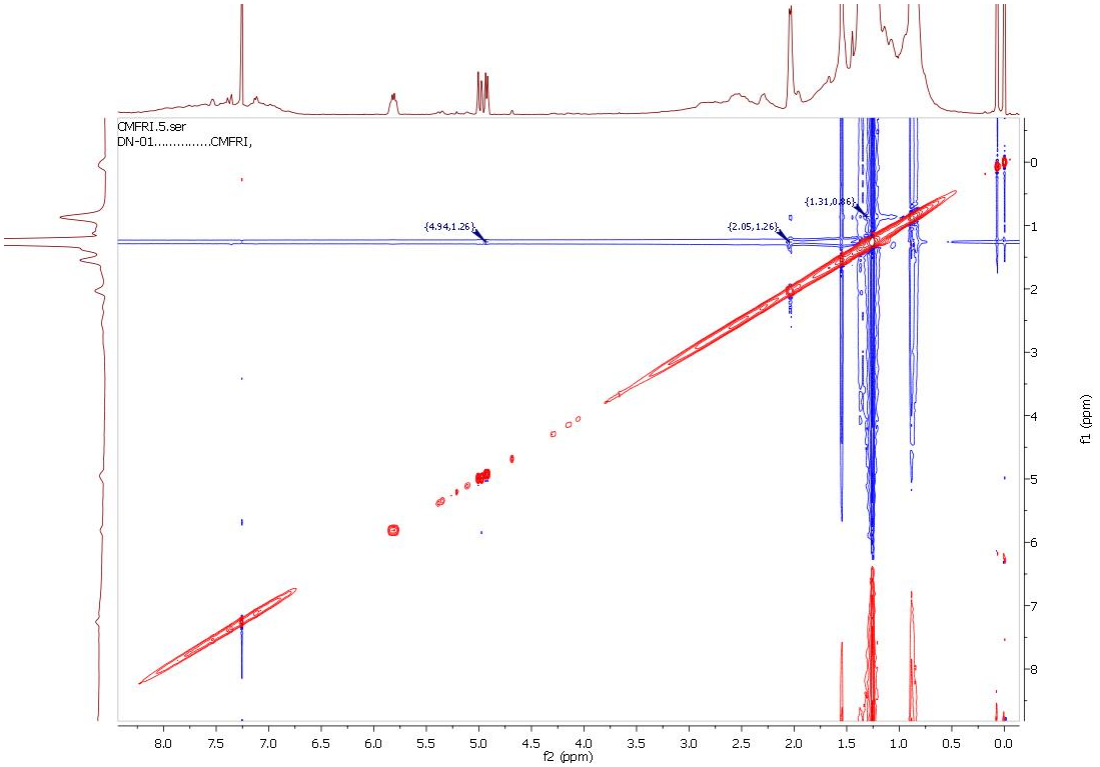
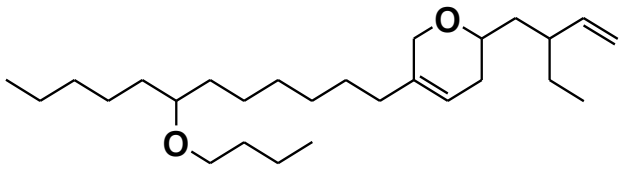


Fig. 9.6.8. NOE spectrum of 2-(but-3-enyl)-tetrahydro-5-nonyl-2H-pyran

9.2.4.2. Structural characterization of COMPOUND II (LD - 56)

COMPOUND II (LD-56)

5-(7-Butoxydodecyl)-2-(2-ethyl-3-butenyl)-3, 6-dihydro-2H-pyran



Sample yield	84 mg (0.42 %*)
Physical description	White semi-solid
Molecular formula	$C_{27}H_{50}O_2$
Molecular weight	406.7592

* % yield based on starting *Loligo duvaucelli* extract

The spectroscopic characterization of the pure and active compound 5-(7-butoxydodecyl)-2-(2-ethyl-3-butenyl)-3, 6-dihydro-2*H*-pyran are discussed in detail below.

5-(7-Butoxydodecyl)-2-(2-ethyl-3-butenyl)-3, 6-dihydro-2*H*-pyran: White semi-solid; UV (MeOH) λ_{max} (log ε): 247 nm (3.21); TLC (Si gel GF_{254} 15 mm; 30% EtOAc/*n*-hexane, v/v) R_f: 0.82; GC (Elite – 5 capillary column 30 m x 0.53 mm i.d.; oven temperature ramp: 60^{o}C for 10 min, rising at 5^{o}C /min to 220^{o}C; 1 mL injection volume/$CHCl_3$) R_t: 7.20 min; Elemental analysis found: C,79.74; H,12.39; O,7.87; IR (KBr, cm^{-1}) ν_{max} 726.29 cm^{-1} (C-H rocking), 1376.26 cm^{-1} (C-H rocking), 1454.38 cm^{-1} (C-H bending of alkanes), 1320 cm^{-1} (C-O stretching) ,1684 cm^{-1} (C=C stretching), 2923.22 cm^{-1} (C-H stretching of alkane), 2955.04 cm^{-1} (C-H stretching of alkane); ^{1}H NMR (500 MHz, Chloroform-d) δ 5.81 (ddt, J = 16.9, 10.2, 6.7 Hz, 1H), 5.38(t, 1H), 3.52(dt, 1H, J=11.2, 6.5), 2.28(m, 2H), 1.83(m, 2H), 1.78(m, 2H), 1.48(m, 2H), 1.01(t, 3H), 0.88(t, 3H), ^{13}C NMR (125 MHz, $CDCl_3$) δ 153.20, 140.76, 121.72, 114.05, 71.82, 56.78, 56.17, 50.15, 42.33, 39.80, 39.53, 37.26, 36.52, 36.20, 35.79, 31.92, 31.68, 29.70, 28.24, 28.02, 24.30, 23.83, 22.82, 21.10, 19.40, 18.72, 14.11, 11.87; ^{1}H-^{1}H-COSY and HMBC data (details under the Table 9.5); HRESIMS *m/e:* ($C_{27}H_{50}O_2$): 406.7592 (calcd. for $C_{27}H_{50}O_2$ 406.1242). The mass fragmentation is shown in Fig. 9.7.

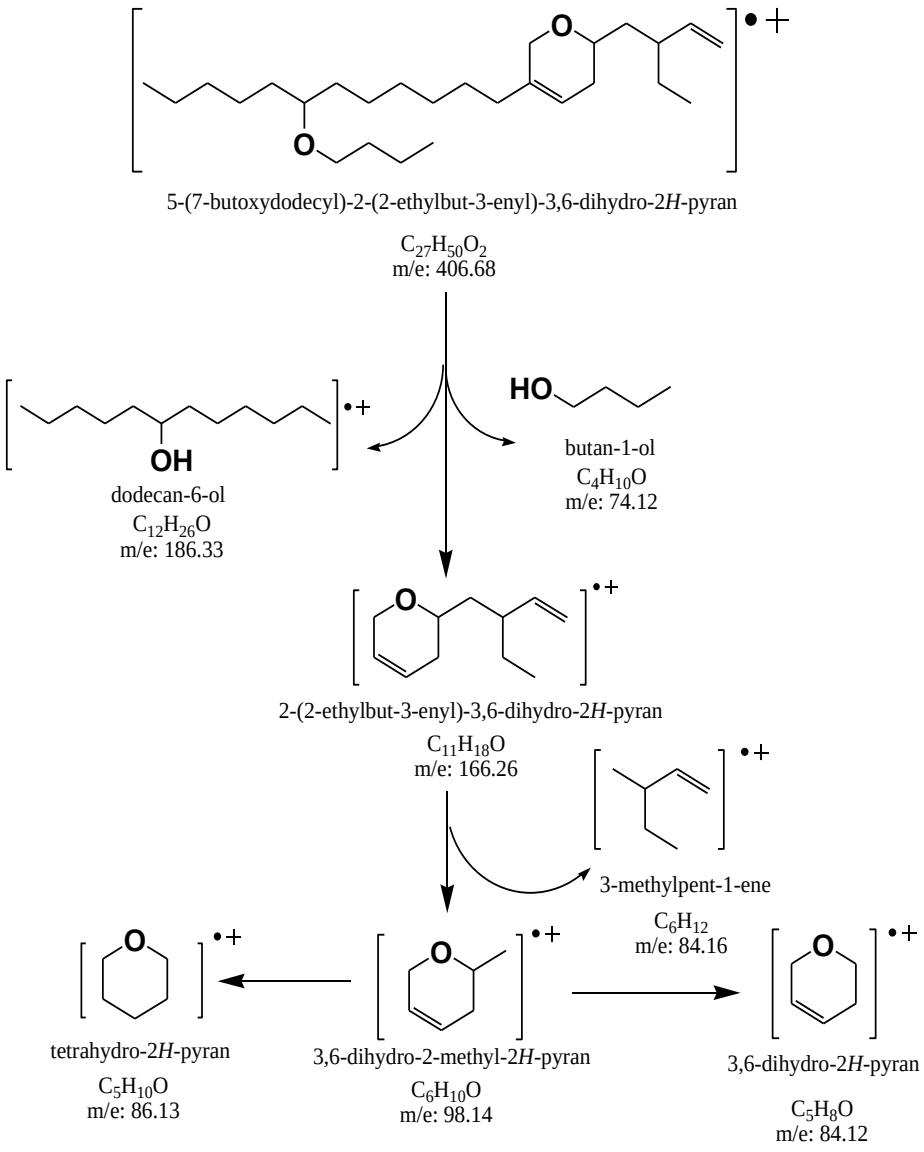


Fig. 9.7. Mass fragmentation pattern of 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3,6-dihydro-2H-pyran

The spectroscopic characterization of the pure and active compound, 5-(7-Butoxydodecyl)-2-(2-ethyl-3-butenyl)-3, 6-dihydro-2H-pyran are discussed as follows. The compound 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3, 6-dihydro-2H-pyran, a new derivative of the substituted pyran was isolated as white semi solid upon chromatography over silica columns/TLC. The presence of 2-(2-ethylbut-3-

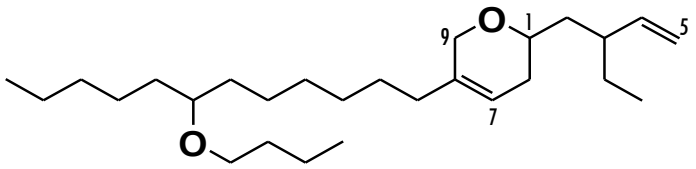
enyl)-3, 6-dihydro-2H-pyran, and 6-butoxydodecane groups have been confirmed by detailed NMR and mass spectroscopic experiments. The ^{1}H NMR in conjugation with ^{13}C-NMR recorded the presence of the methine groups CH at δ 3.52, 2.04, 1.08, 5.82, and 5.38 ppm. The latter two methane group has been assigned to be due to the presence of olefinic double bond, whereas the methine group appeared at δ 3.52 is a part of the pyran ring system, and the downfield shift of the –CH group is due to the presence of alpha -O-C- from the methane group. The methylene group at δ 5.01 and 4.98 ppm appeared significantly downfield due to their presence at the terminal (as $=CH_2$) part of 3-methylpent-1-ene side chain attached to the pyran ring system. The methylene group at δ 4.32 ppm appeared significantly downfield, and was assigned to be the part of the 6-butoxydodecane side chain. The querternary carbon appeared at δ 153.2 ppm has been established as the junction point between the pyran ring system and 6-butoxydodecane side chain as established by detailed spectroscopic experiments. The other methylene groups at δ 2.08, 1.84, 1.78, and 1.31 ppm also corroborated with the deduced structure and part of the side chain attached with the pyran group. This has been confirmed by the strong ^{1}H-^{1}H-COSY correlation between these methylene groups. The downfield shift of the $–CH_2$ protons at δ 2.18 ppm also established the presence of alpha -C=C moiety belonging to the pyran ring system. Peaks at C-4 (δ 140.76) and C-8 (δ 153.20) indicate the presence of olefinic groups. Protons H-2 through 4 afforded HMBC correlation with C-3, 3', and C-4 indicates the presence of 3-methylpent-1-ene system attached to the substituted pyran ring system. The methylene group protons appeared at δ 2.28 and 2.17 ppm, are assigned to be the part of 3,6-dihydro-2H-pyran system. The presence of the 6-butoxydodecane and 3-methylpent-1-ene side chain along with the pyran moiety has been confirmed by ^{1}H-^{1}H COSY experiments. The H-10 (δ 2.08 ppm) showed ^{1}H-^{1}H COSY correlation with H-11 (δ 1.84), which showed HMBC correlation with C-12 (δ 31.92) (Table 9.5). The carbon at C-22 (δ 56.72) exhibited DEPT signal for methylene group, and its downfield shift demonstrates the presence of highly

electronegative group at its close proximity. The bunches of carbons at C-10, 11, 12, 13, 14, and 15 are linearly aligned as established by ^{1}H-^{1}H COSY correlation results. The ^{1}H-^{1}H COSY correlations at H-22 (δ 4.32) with H-23 (δ 1.52), H-24 (δ 1.03), and H-25 (δ 0.87) are the prominent ones, which also established the presence of oxobutane system. In the ^{1}H–^{1}H COSY spectrum, couplings were apparent as described in the Fig. 9.8.1 support the presence of the established skeleton. These protons at H-4 (δ 5.82), H-6 (δ 2.28) and H-9 (δ 2.18) showed HMBC correlation with C-2 (Fig. 9.8.1), which support the structure of 2-(2-ethylbut-3-enyl)-3,6-dihydro-2H-pyran system. The protons at H-12 (δ 1.78) and H-15 (δ 1.83) exhibited strong HMBC correlation with C-10 (δ 33), which establishes the 6-butoxydodecane side chain attached to the pyran ring system. The ^{13}C NMR spectrum of the purified compound in combination with DEPT experiments indicated the occurrence of 27 carbon atoms in the molecule. The geometrical configuration of the olefinic protons ('E'-type) was further confirmed from the J value (14 Hz) and detailed NOESY experiments.

The olefinic (C=C), and ether (C-O-C) groups have been symbolized by the absorption bands at 1684 and 1320 cm^{-1} respectively. The characteristic IR absorption spectra at 726.29 cm^{-1} (C-H rocking), 1376.26 cm^{-1} (C-H rocking), 1454.38 cm^{-1} (C-H bending of alkanes), 1320 cm^{-1} (C-O stretching) ,1684 cm^{-1} (C=C stretching), 2923.22 cm^{-1} (C-H stretching of alkane), 2955.04 cm^{-1} (C-H stretching of alkane) also support the structure of 5-(7-butoxydodecyl)-2-(2-ethyl-3-butenyl)-3, 6-dihydro-2H-pyran. Its mass spectrum exhibited a molecular ion peak at m/e 406, which in combination with its ^{1}H and ^{13}C NMR data (Table 9.5) indicated the elemental composition of $C_{27}H_{50}O_2$. The mass spectrum of 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3, 6-dihydro-2H-pyran, depicted in (Compound II), shows three major peaks with the ion peak at m/e 98 of high abundance. The molecular ion peak at m/e 406 appeared to undergo elimination of $C_4H_{10}O^+$ (m/e 74, butan-1-ol) and $C_{12}H_{26}O^+$ (m/e 186, dodecan-6-ol) to yield m/e 166 ($C_{11}H_{18}O^+$,2-(2-ethylbut-3-enyl)-

3, 6-dihydro-2H-pyran), which undergoes fragmentation to afford the fragments with m/e 98, 84, 86 etc. The base peak appeared to be due to 3, 6-dihydro-2-methyl-2H-pyran (m/e 98, $C_6H_{10}O^+$). These signature peaks established the presence of the 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3,6-dihydro-2H-pyran moiety.

Table 9.5 NMR spectroscopic data of 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3, 6-dihydro-2H-pyran in $CDCl_3$.[a]



Carbon no.	^{13}C NMR (DEPT)	H	δ^1H NMR (int., mult., J in Hz)[b]	^1H-^1H COSY	HMBC (^1H-^{13}C)
1	71.82	1H	3.52(dt,1H,J=11.2,6.5)	H-2,6	
2	31.85	2H	1.49(m,2H)	H-3	C-3,3'
3	39.80	3H	2.04(m,1H)	H-3',4	C-4
3'	27	3'H	1.15(m,2H)	H-3'	C-4,3''
3''	11.87	3''H	1.01(t,3H)		
4	140.76	4H	5.82(ddt,1H,J=16.9,10.2,6.7)	H^b-5	C-2
5	114.05	5H[a]	5.01	H-4	
		5H[b]	4.98		
6	50.15	6H	2.28(m,2H)	H-1,7	
7	121.72	7H	5.38(t,1H)		C-8
8	153.20				
9	42.20	9H	2.18(s,2H)		C-1,2
10	33	10H	2.08(t,2H,J=6.5)	H-11	C-7
11	37.26	11H	1.84(m,2H)	H-12	
12	31.92	12H	1.78(m,2H)	H-13	C-10
13	29.70	13H	1.31(m,2H)	H-14	
14	24.30	14H	1.57(m,2H)		C-13,11
15	28.24	15H	1.83(ddq,2H,J=18.9,9.4,6.2,4.5)	H-16	
16	56.17	16H	1.08(m,1H)	H-17	
17	28.02	17H	1.48(m,2H)		C-15,18
18	23.82	18H	1.10(m,2H)	H-19	
19	22	19H	0.99(m,2H)	H-20	C-20,21
20	19.40	20H	0.92(m,2H)	H-21	
21	14.11	21H	0.87(t,3H)		
22	56.78	22H	4.32(t,2H,J=7.3)	H-23	C-16,23
23	36.20	23H	1.52(m,2H)	H-24	
24	19.40	24H	1.03(m,2H)	H-25	C-25
25	14.68	25H	0.87(t,3H)		

[a] NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometers.

[b] Values in ppm, multiplicity and coupling constants (J¼ Hz) are indicated in parentheses. Assignments were made with the aid of the ^1H-^1H COSY, HSQC, HMBC and NOESY experiments.

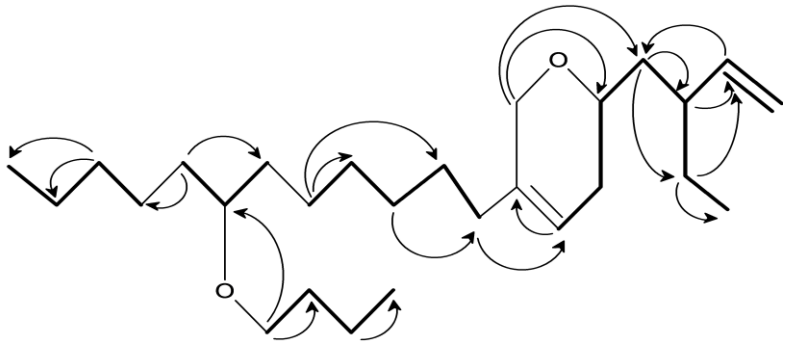


Fig. 9.8.1. ^{1}H—^{1}H COSY and HMBC correlations of 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3, 6-dihydro-2H-pyran. The key ^{1}H-^{1}H COSY couplings have been represented by the bold face bonds; The HMBC couplings are indicated as double barbed arrow

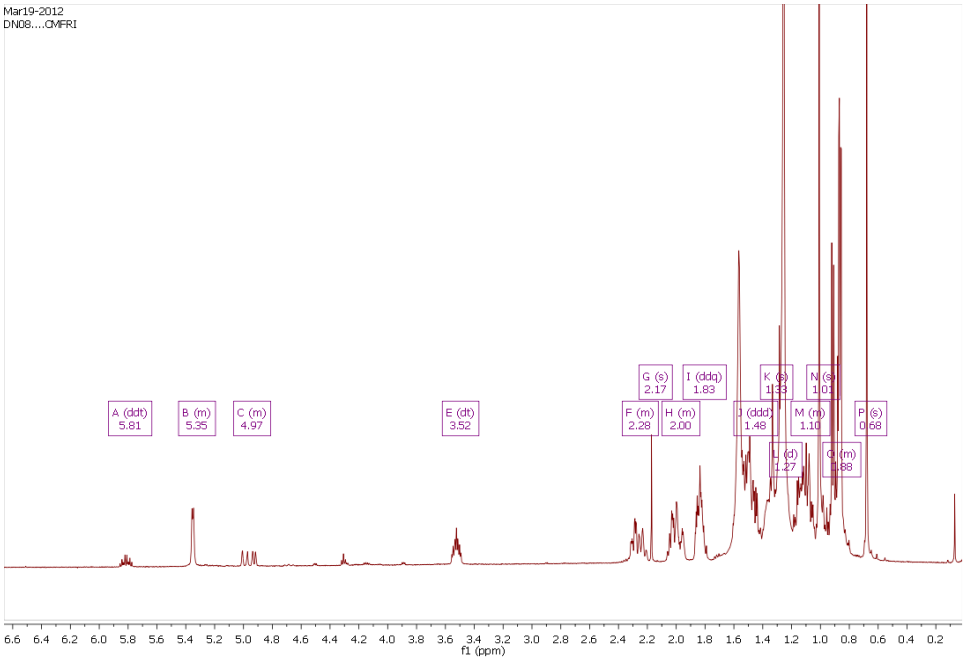


Fig. 9.8.2. Proton NMR spectrum of 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3,6-dihydro-2H-pyran

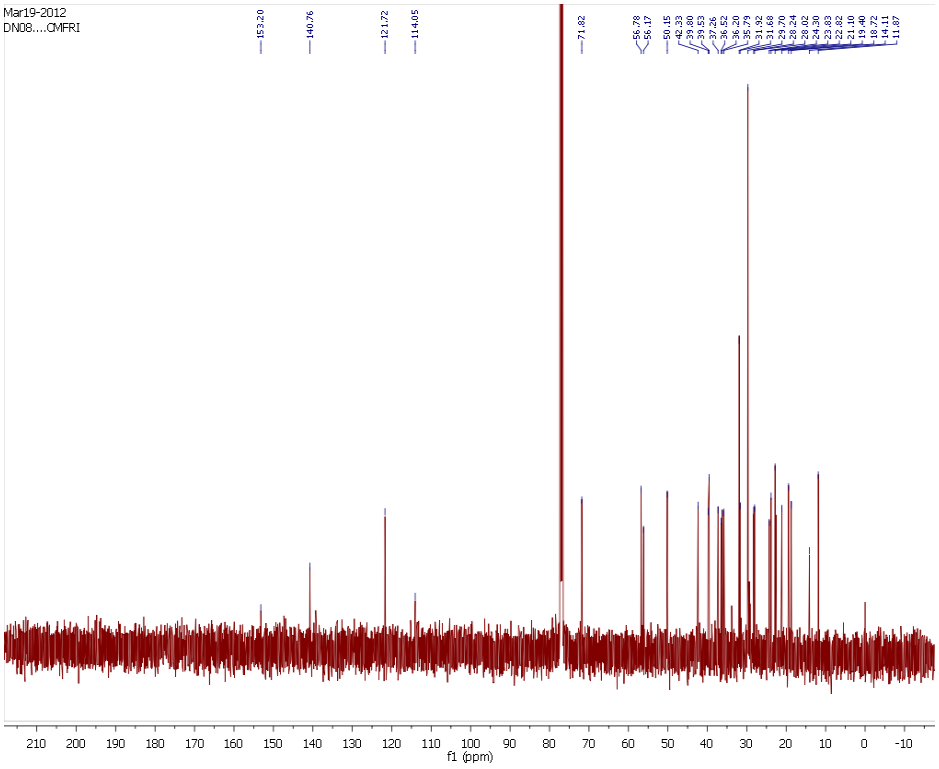


Fig. 9.8.3. ^{13}C NMR spectrum of 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3,6-dihydro-2H-pyran

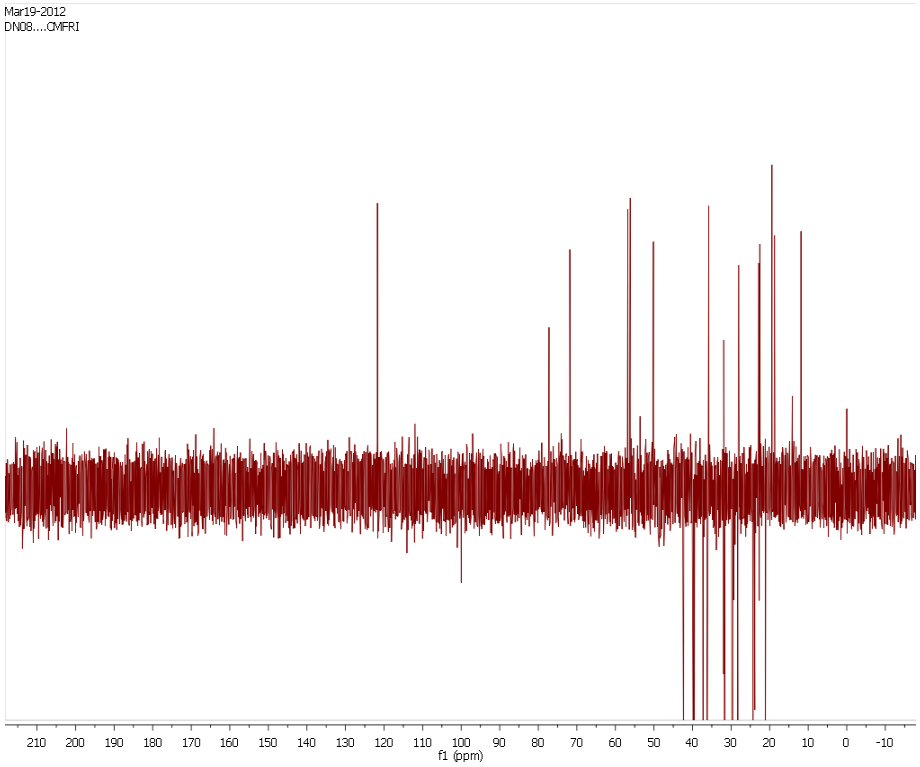


Fig. 9.8.4. DEPT spectrum of 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3,6-dihydro-2H-pyran

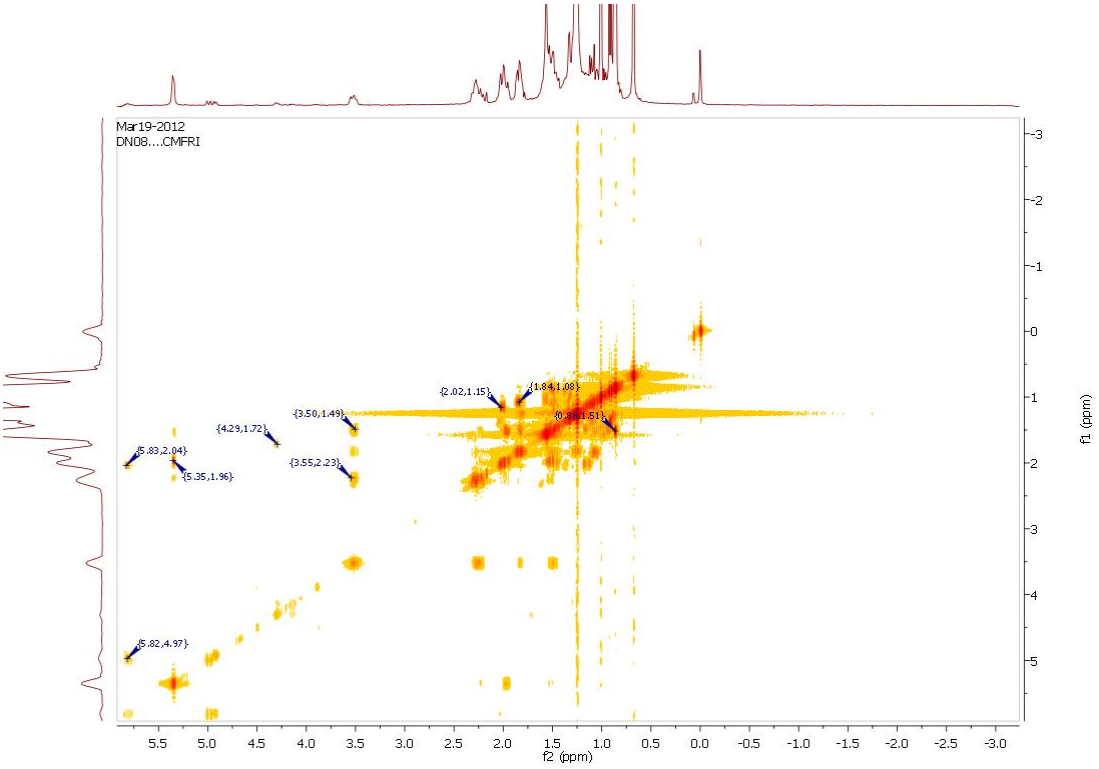


Fig. 9.8.5. ^{1}H–^{1}H COSY spectrum of 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3,6-dihydro-2H-pyran

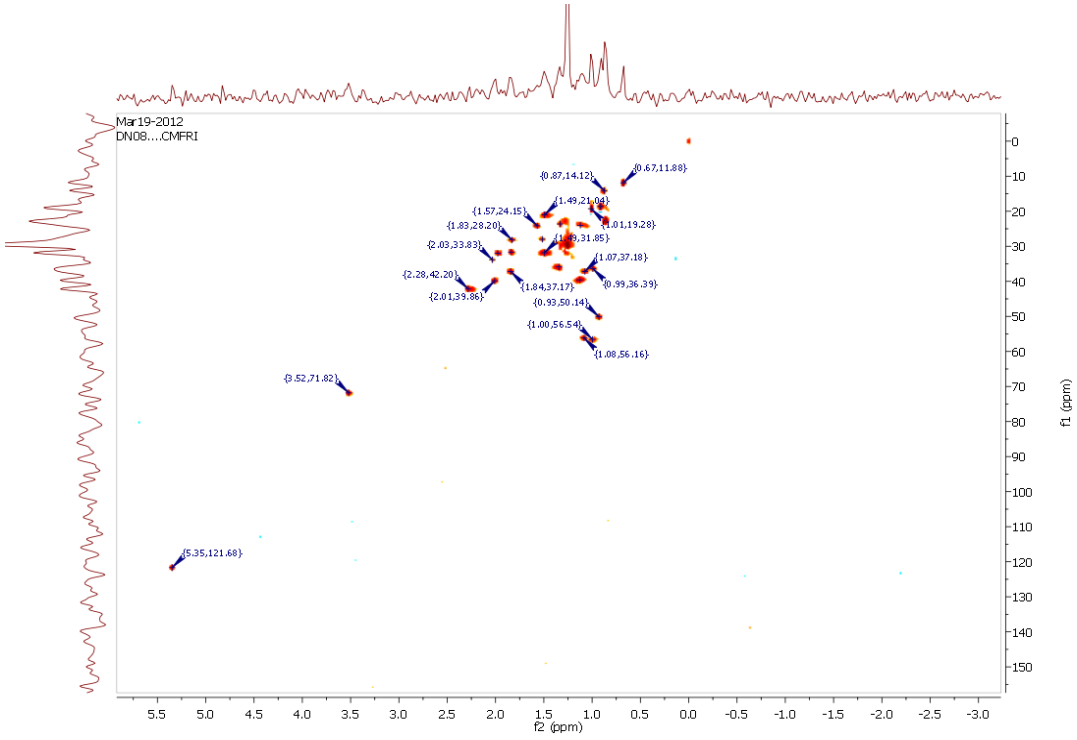


Fig. 9.8.6. HSQC spectrum of 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3,6-dihydro-2H-pyran

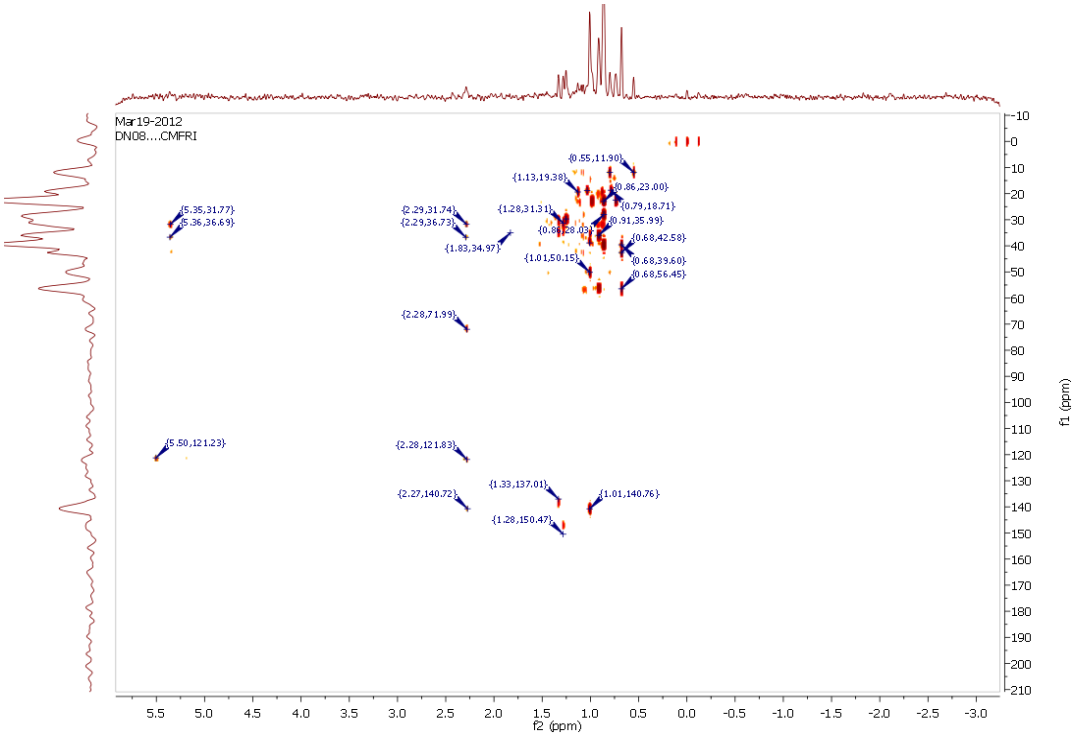


Fig. 9.8.7. HMBC spectrum of 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3,6-dihydro-2H-pyran

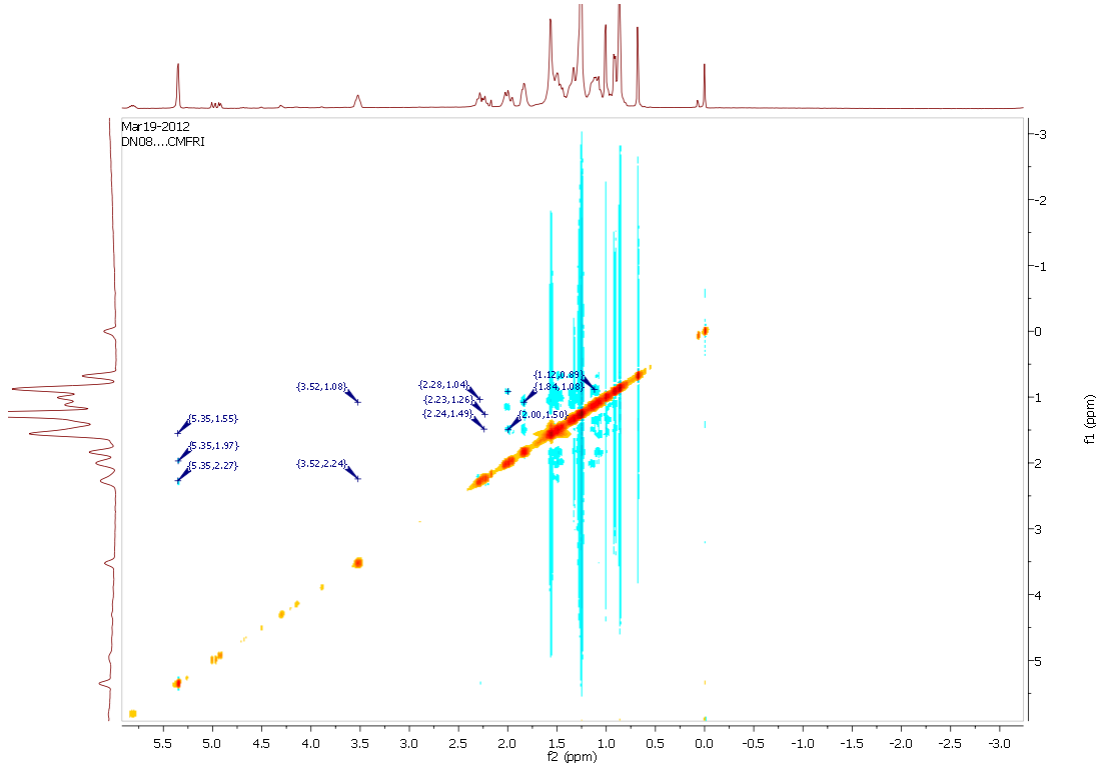
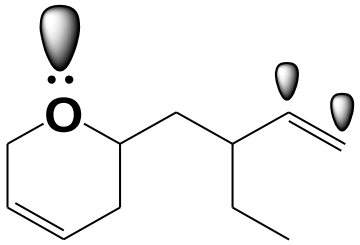


Fig. 9.8.8. NOE spectrum of 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3,6-dihydro-2H-pyran

The pyran ring system is widely present in the animal and plant kingdom and possesses diverse pharmacological activities. Pyran is six-membered oxygen heterocycle with two double bonds. Out of the five carbon atoms of the pyran ring, four are sp^2 carbons and one is sp^3 hybridised. The pyran ring is not a true aromatic ring. Partially reduced pyrans such as III and IV are always named using the suffix 2H as established in the present study. The compounds with the pyran ring system have been used as precursors for the synthesis of pharmacologically active compounds such as HIV protease inhibitors, antifungals, cardiotonics, anticonvulsants, antimicrobials, and anti-tumour agents (Goel & Ram, 2009). Microbially derived 2-pyranones, obtained from fungi of various genera, are found to display a wide range of cytotoxic, neurotoxic and phytotoxic properties. Earlier studies reported the isolation of two pyrans, dendroflorin and denchrysan A, from the whole plant of *Dendrobium* genus, used as a health-food (Chen *et al*., 2008). The bufadienolide class of compounds constitutes the core skeleton of structurally unique 2-pyranone natural products (Deepak *et al*., 1997) in which a steroid moiety is attached at position five of the lactone ring. This class of compounds is widely used in traditional remedies for the treatment of several ailments, such as infections, rheumatism, inflammation and disorders associated with the central nervous system. Polyhydroxystyryl-pyran-2-one derivative, phelligridin B, together with a new pyranopyrandione, have been isolated from the fruit bodies of the Chinese medicinal fungus *Phellinus igniarius* (Barry *et al*., 1997). Two new dihydrostyrylpyrones and a styrylpyrone, together with a known styrylpyrone, have been isolated from the ethyl acetate-soluble fraction of an aqueous ethanol (1:4) extract of the herb *Polygala sabulosa*, which is used as a topical anaesthetic in folk medicine (Goel & Ram, 2009).

Among the compounds isolated from *Loligo duvauceli*, 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3, 6-dihydro-2H-pyran was found to be possess potent antioxidative activity due to the presence of 2-(2-ethylbut-3-enyl)-3,6-dihydro-2H-

pyran group. Other compounds with no pyran moiety did not show equivalent antioxidative activity proving that the pyran group has major role in the target bioactivity to neutralize reactive oxygen species.



2-(2-ethylbut-3-enyl)-3, 6-dihydro-2H-pyran group

The multiple olefinic (two numbers) and pyran groups serve as the potential electronegative centre of the compound.

9.3. Conclusions

The compounds belonging to pyran derivatives such as 2-(but-3-enyl)-tetrahydro-5-nonyl-2H-pyran and 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3,6-dihydro-2H-pyran were isolated from the EtOAc:MeOH extract of *Loligo duvauceli*, and were demonstrated to possess potential anti-inflammatory and antioxidative properties. 2-(But-3-enyl)-tetrahydro-5-nonyl-2H-pyran (Compound I) showed 37.4 % COX-2 inhibition activity, 33.01 % LOX-5 inhibition activity (both at 0.5mg/ml) with 68.6 % free radical scavenging activity. The compound II, exhibited 36.2 % COX-2 inhibition activity, 30.1 % LOX-5 inhibition activity (both at 0.5mg/ml) with 71.7 % free radical scavenging activity. The anti-inflammatory potential of these compounds derived from *Loligo duvauceli* are proved to be comparable to the synthetic anti-inflammatory agents, aspirin and indomethacin. It is therefore, these compounds containing EtOAc:MeOH extract of *Loligo duvauceli* may enrich the anti-inflammatory potential of sardine oil and this study was conducted in the following chapter.

Chapter 10

PREPARATION OF ENRICHED PUFA FORMULATION FOR USE AS NUTRACEUTICAL AND AQUAFEED SUPPLEMENTS

Contents

Background

Every tissue in human body produces bioactive eicosanoids having a wide range of physiological actions, immune and inflammatory responses, cardiovascular, renal and neural functions, and reproduction process. The purified methyl esters from *Sardinella longiceps*, is abundant in C_{20}-C_{22} *n*-3 PUFAs, particularly eicosapentaenoic acid (20:5*n*-3, EPA) and docosahexaenoic acid (22:6*n*-3, DHA), which are the precursors of anti-inflammatory resolvins (E- and D-series). Arita et al. (2005) reported that the anti-inflammatory effect of methyl esters was due to the anti-inflammatory compound termed as resolvins (resolution- phase interaction product) E1, which are produced from EPA. In recent days, research on exploring the sources of the long-chain *n*-3 PUFAs, *viz.*, DHA and EPA for use in nutrition have received considerable attention. These PUFAs, which are usually low in abundance in human, are regarded as essential and must be supplied in diet. It is therefore necessary to supplement these essential fatty acids by the external means. Earlier chapter (*Chapter 7*) detailed the preparation of stabilized PUFA concentrate using sardine fatty acid methyl esters as the starting material.

The sardine methyl esters in its pure form are embedded with unsaturated fatty acid matrix. This can potentially result in changes in its physicochemical characteristics (formation of high risk decomposition substances *viz.*, free radicals, small molecular weight aldehydes and ketones) during several processing steps and storage, and thereby may potentially alter its anti-inflammatory properties. It has been well documented that the reactive oxygen species (ROS) are important mediators that initiate and propagate inflammatory responses by stimulating the

release of pro-inflammatory cytokines such as interleukin-1b (IL-1b) and tumour necrosis factor (TNF-α) (Geronikaki & Gavalas 2006). It was also found that ROS induced by activated neutrophils, eosinophils, monocytes and macrophages during the inflammation process leads to tissue injury by damaging macromolecules and effecting the lipid peroxidation of membranes (Gutteridge, 1995). This indicates that the free radical scavengers might be a useful means of attenuating the inflammatory effects. The present work demonstrated the functional role of the extracts derived from the marine cephalopod species *Loligo duvauceli* (shortlisted as potential candidate species based on the results obtained in the earlier chapter (*Chapter 9*) with antiradical and anti-inflammatory active principles to enrich the anti-inflammatory potential of the purified sardine methyl esters. *In vivo* anti-inflammatory activity of the enriched PUFA formulation was studied by the carrageenan-induced mice paw edema model.

10.1. Materials and Methods

10.1.1. Chemicals, Reagents and Instrumentation

Potassium phosphate, Tween 20, leuco-2, 7-dichlorofluorescein diacetate, hematin, phenol, arachidonic acid, lipoxidase extra pure (LOX-5), cyclooxygenase 1 (from sheep, COX-1), cycloxygenase-2 (human recombinant, COX-2), and carrageenan were procured from Sigma-Aldrich Chemical Co. Inc. (St. Louis, MO).

An electronic micrometer (aerospace; 0-25 mm range, least count: 0.001 mm) was used for measuring the paw thickness of mice.

10.1.2. Preparation of an Enriched Anti-Inflammatory PUFA Formulation from Purified Sardine Oil

The purified sardine methyl esters (PO) with the antioxidant combination of the red seaweed extracts of *K. alvarezzi*, *H. musciformis* and *J. rubens* at 1% (POC; *Chapter 7; Section*) was blended with *Loligo duvauceli* extract (0.5%, w/w) and designated as POI.

10.1.3. *In vivo* Anti-Inflammatory Activity

The *in vivo* carrageenan-induced mice paw edema experiment was carried out as previously described (Winter *et al.*, 1962; Nobre *et al.*, 2013) with modifications (*Chapter 9*). The animals were divided into 5 groups of 6 animals each. The methyl esters was mixed with 1% Tween 80 and orally administered at 5 mg/kg. The other groups were injected with either the reference drug (aspirin, 5 mg/kg, orally) or vehicle (Tween 80). After thirty minutes, 1% solution of carrageenan in saline (0.1 ml/mice) was injected subcutaneously into the right hind paw. Paw volumes were measured at 0, 2, 3, 4, 5 and 6 h after injection, and the thickness of the edema was measured with an electronic micrometer (aerospace; 0-25 mm range, least count: 0.001 mm).

10.2. Results and Discussion

10.2.1. Enrichment of Purified Sardine Fatty Acid Methyl Esters for use as Nutraceutical Supplements

To evaluate the anti-inflammatory activity, one of the well-established acute inflammatory models *in vivo*, carrageenan-induced paw edema model in mice was opted. The development of carrageenan induced edema is biphasic, the first phase is attributed to the release of histamine, 5- HT and kinins, and occurs within an hour of injection, and is partly due to the trauma of injection, while, the second phase is related mainly to prostaglandins (PGs), which is measured around 3h (Vane & Booting, 1987). The intra-plantar injection of 0.1 ml of carrageenan (1% solution of carrageenan in saline) into the mice hind paw induced progressive increasing edema volume in the control group as inflammatory response as early as 0 - 6 h. Pre-treatment with the purified *n*-3 rich fatty acid esters at a dose of 5 mg/kg showed significant inhibition of the paw swelling caused by carrageenan after 5 h (Table 10.1). Arita et al. (2005) reported that the anti-inflammatory effect of fish oil appeared to be due to the anti-inflammatory compounds termed as resolvins (resolution- phase interaction product) E1, which is produced from EPA. The principle function of resolvin E1 was reported to be as a counter-regulator to proinflammatory mediators and acute inflammatory processes. It is of note that the

PO exhibited maximum inhibition of 58 % at the 6^{th} h of the experiment. This showed that PO significantly inhibited the paw edema induced by carrageenan in the second phase, which further suggest a possible inhibition of COX synthesis by the oil, because the carrageenan inflammatory model reflects the actions of prostaglandins. This effect is similar to that produced by non steroidal anti inflammatory drugs such as aspirin, which primarily inhibit the COX enzymes. Under the similar experimental conditions, the anti-inflammatory effect of the purified fatty acid esters added with seaweed extracts (POC) were found to be higher as compared to the PO at 5^{th} and 6^{th} h (Table 10.1). However, POI showed significantly higher inhibition of mice paw edema ($p<0.05$). Interestingly, POI seemed to be more effective than POC or PO from the 4^{th} h of the study. Clinical studies have reported that fish oil supplementation has beneficial effects in rheumatoid arthritis, inflammatory bowel disease and some asthmatics, thereby supporting the idea that the *n*-3 PUFAs in fish oil are anti-inflammatory (Calder 2008). Recent studies have identified novel families of mediators formed from *n*-3 fatty acids EPA and DHA, termed as E- and D-series resolvins, respectively. These mediators are derived from EPA and DHA produced via the cyclooxygenase and lipoxygenase reactions, and are considered to be anti-inflammatory (Calder 2006). The LC *n*-3 PUFAs have been demonstrated to modulate the inflammatory reaction especially by a competition mechanism at the level of lipoxygenase and cycloxygenase, thereby resulting in a reduced release of pro-inflammatory arachidonic acid metabolites. These may explain many of the anti-inflammatory actions of *n*-3 fatty acids that have been described in an earlier study (Gilroy et al. 2004). The *n*-6 fatty acids (such as C20:4*n*-6) are metabolized by cycloxygenases to prostaglandins PGG_2 and then PGH_2 that serves as a substrate for a series of downstream synthesis to give rise to the inflammatory PGs. In contrast, anti-inflammatory resolvins biosynthesized from EPA (18*R*-HEPE series) and DHA (17R-HDHA series) lead to the inhibition of the recruitment of the potent inflammatory mediators *in vitro* and *in vivo* (Gilroy *et al.*, 2004).

Table 10.1 *In vivo* anti-inflammatory activities of purified sardine oil blended with additives

Samples	Paw edema (mm)	Paw edema (mm)	Difference in paw edema (%)*	(% inhibition)	Paw edema (mm)	Difference in paw edema (%)*	(% inhibition)	Paw edema (mm)	Difference in paw edema (%)*	(% inhibition)	Paw edema (mm)	Difference in paw edema (%)*	(% inhibition)	Paw edema (mm)	Difference in paw edema (%)*	(% inhibition)
	0h	2h	2h	2h	3h	3h	3h	4h	4h	4h	5h	5h	5h	6h	6h	6h
CONTROL	1.16	2.49	114.66		2.51	116.38		2.52	117.24		2.53	118.1		2.54	118.97	
Standard	1.46	2.22	52.05	54.6	2.05	40.41	65.28	1.99	36.3	69.04	1.92	31.51	73.32	1.88	28.77	75.82
LD	1.46	2.35	60.96	46.83	2.11	44.52	61.75	2.05	40.41	65.53	2.02	38.36	67.52	2.03	39.04	67.18
PO	1.24	2.16	74.19	35.29	2.22	79.03	32.09	2.18	75.81	35.34	2.11	70.16	40.59	1.86	50	57.97
POC	1.36	2.3	69.12	39.72	2.21	62.5	46.3	2.14	57.35	51.08	2.05	50.74	57.04	1.89	38.97	67.24
POI	1.44	2.41	67.36	41.25	2.35	63.19	45.7	2.05	42.36	63.87	1.69	17.36	85.3	1.66	15.28	87.16

LD - *Loligo duvauceli* extract; PO - Purified Oil; POC - purified oil with the antioxidant combination at 1% (POC; *Chapter 7*); POI – Purified oil blended with *Loligo duvauceli* extract (0.5%, w/w, POI)

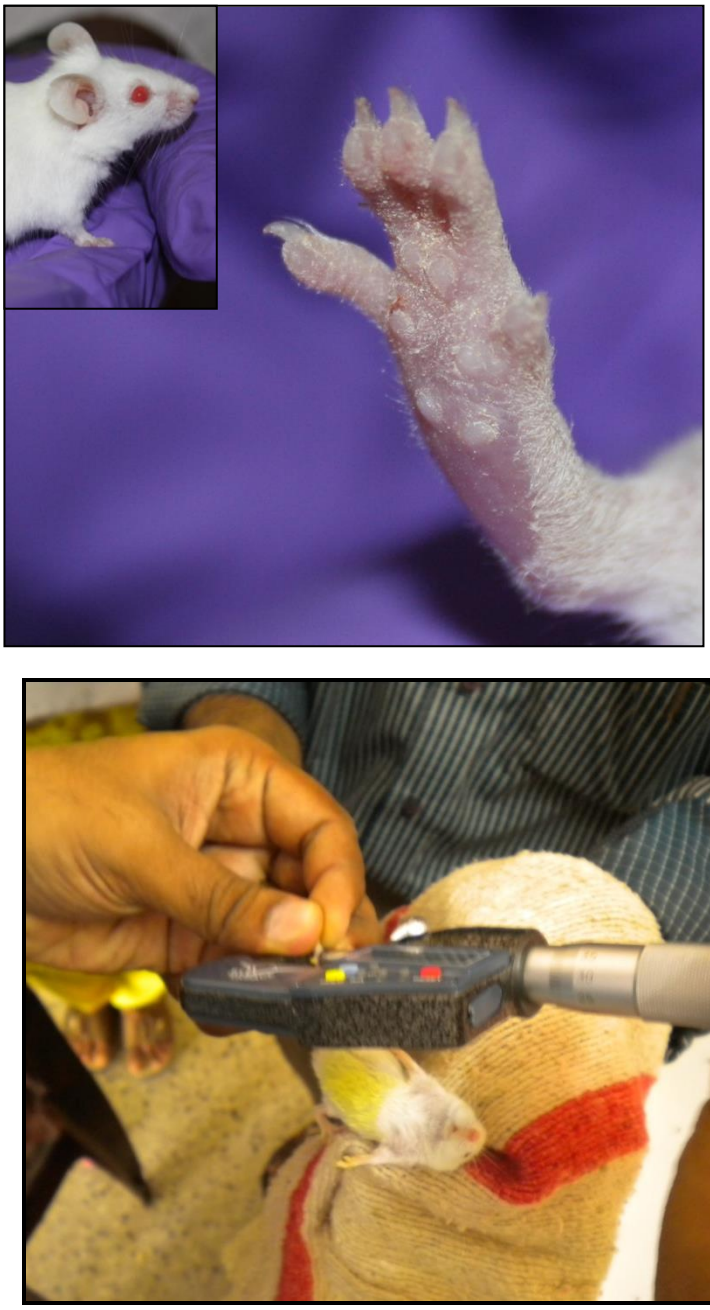


Fig.10.1. Photographs showing **(A)** mice paw edema after 30 min of carrageenan injection (inset: BALB/C mice); (**B**) The measurement of paw edama using a micrometer (aerospace, 0-25 mm range, least count: 0.001 mm)

Fig. 10.2. Purified sardine FAME capsules stabilized with the marine antioxidant blend and enriched with *Loligo duvauceli* extract

10.3. Conclusions

The evolution of new fish oil products based on refined oil or its concentrated derivatives of specific essential long chain fatty acids are constantly being introduced into the market. This study successfully enriched the polyunsaturated fatty acids in its ester form from the marine body oil from the widely available species, *Sardinella longiceps*, and further demonstrated the anti-inflammatory property of the PUFA enriched formulation by using the cephalopod, *Loligo duvauceli* extract. The addition of *Loligo duvauceli* significantly enhanced the anti-inflammatory property of the PUFA enriched purified oil, which has been demonstrated by the carrageenan-induced paw edema model study in mammalian subjects. The anti-inflammatory activity of the PUFA enriched formulation was found to be significantly higher after 4 h of carrageenan injection. It is therefore, the PUFA enriched sardine oil supplemented with *Loligo duvauceli* extract may be used as a nutraceutical supplement human health and nutrition, and is an effective alternative to the synthetic non steroidal anti-inflammatory drugs (NSAIDs) for use against mammalian inflammatory diseases.

It is of note that these *n*-3 PUFAs in adequate amount are essential in the diet of marine finfish for larval and broodstock nutrition under mariculture, because of

the inability of the fish to synthesize *de novo* these fatty acids in adequate levels from precursor molecules. The PUFAs are therefore, are one of the most critical inputs to be supplied during larval growth due to their use for a cascade of growth and development parameters. The above derived fatty acid enriched and stabilized form of sardine oil in its ester form can therefore be used as an effective PUFA supplement for use in the aquafeed and feedstuff industry. The lipid peroxidation of the PUFA supplement in aquafeed may lead to byproducts that negatively affect the quality of the aquafeed. The oxidized fatty acids in the aquaculture diet can affect the carcass quality and fatty acid composition of the aquacultured species. It is evident from the earlier chapter (*Chapter 7*) that the purified methyl esters supplemented with marine antioxidants were able to retain the stability of long chain polyunsaturated fatty acids, especially, EPA and DHA. It is therefore, that the stabilized purified methyl esters prepared in the present work can be used as aquafeed supplement, and could be able to maintain the palatability, odor and health benefits of the aquafeed for a prolonged period.

Chapter 11

SUMMARY

The long chain *n*-3 polyunsaturated fatty acids (PUFAs) with C_{20-22} acyl chain length, particularly eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3), were reported to have various important physiological roles in health, and in the inflammatory process. Whilst saturated and monounsaturated fatty acids may be synthesized in the body, PUFAs cannot be synthesized *de novo* due to the lack of essential enzymes required to synthesize PUFAs in adequate levels from the precursor fatty acids, and therefore, must be externally supplied in the diet. Since these pharmacological and physiological functions drew attention to these essential fatty acids, the production of PUFA-rich fish oil as a food material is a growing research area. The PUFAs are currently in demand in the pure form in nutraceutical and aquafeed formulations, and are being actively studied to understand their potential role in human health.

There are reports of concentrates of PUFA from marine alga, fish oil (Guil-Guerrero *et al*., 2007), and edible oils (López-Martínez *et al*., 2004). Also fishery by-products/processing waste and low value catches have been utilized for concentrating PUFA (Zuta *et al*., 2003). A survey of the literature revealed a diverse range of methods for enrichment and purification of LC-PUFAs.

PUFAs are found to be widely available in various marine fish species such as *Trichiurus lepturus*, *Leiognathus splendens*, *Sardinella longiceps* and *Epinephelus diacanthus*, which were evaluated for the comparative analysis of their spatial (Southwest and Southeast coast of India) and seasonal (pre-monsoon, monsoon and post-monsoon seasons) disparities in the fatty acid composition. However, oil sardine (*Sardinella longiceps*) is a low-value fish, and the crude oil derived from this species is locally available as a byproduct. A higher concentration of PUFAs (31 %) particularly EPA (17.1 %) in sardine oil is an added advantage. The production of

highly refined marine originated lipids enriched with polyunsaturated fatty acids by using sardine oil as the starting material will be of greater commercial importance.

The present study demonstrated that *Sardinella longiceps* is a valuable source of the essential PUFAs, especially, EPA and DHA, for human diet as well as the valuable raw material for extraction of oil in view of commercial exploitation. It has been demonstrated that *Sardinella longiceps* harvested from the Southwest coast, especially during monsoon and post-monsoon seasons, showed its superiority over other studied species when EPA, DHA, total polyunsaturated fatty acids, and fatty acid based nutritional indices were considered. Insignificant variations in fatty acid based nutritional indices, higher lipid yield, maximum EPA content and lower saturated fatty acids of the oil sardines collected during the monsoon season (Southwest coast) compared to the oil sardines collected during post-monsoon have been noted. This demonstrated the importance of this low-value fish collected during monsoon season from the Southwest coast for commercial use, particularly for marine fish oil industries. In addition, oil sardine collected from the Southwest coasts during this period can be considered as an ideal nutritive source due to the optimum balance of the essential nutrients *viz*., amino acids, minerals and vitamins.

An extraction procedure to isolate total lipid with higher *n*-3 fatty acids from the crude sardine oil was identified by comparing the oil characteristics of various extraction modes. The extraction of the *Sardinella longiceps* oil by using the methods of cooking and pressing has been demonstrated to be important to obtain the best quality sardine oil with respect to total yield and *n*-3 fatty acid composition. The fatty acid composition was found to be comparable with the conventional Bligh and Dyer method with respect to polyunsaturated fatty acids (> 26 %), EPA (> 15 %) and DHA (> 4.5%) contents. The oil quality characteristics, which includes a range of lipid oxidation parameters *viz*., free fatty acid (FFA), thiobarbituric acid reactive species (TBARS), peroxide (P.V.), para-anisidine (pA.V.), and the total oxidation values (TOTOX), along with the oxidative stability index (OSI) were compared. The oil obtained by the physical extraction method such as cooking, followed by pressing were found to be efficient to afford a product with greater quality. The oil obtained

by these methods furnished the crude oil with 41, 15.5, 5 & 26.5 % total SFA, EPA, DHA and total PUFA, respectively, which contain the non-triglyceride part, colorants, odorous and toxic compounds, and therefore, it was demonstrated to be unsuitable for human consumption. It is therefore, that subsequent refining and purification have been performed in order to eliminate the unpalatable impurities, and enrich the *n*-3 fatty acids. Refining of the oil by degumming, bleaching and deodorization were found to effectively eliminate the colored and volatile compounds along with other impurities of undesirable nature. Along with the common oil quality parameters, this study also demonstrated the importance of employing the spectroscopic techniques such as FT-IR and ^{1}H-NMR spectroscopy conjugated with ^{13}C NMR methodologies to monitor the finer qualities of the oil at various stages of refining. Significant reduction in the contents of hydrocarbon functionalities of the crude oil was afforded by using 1% phosphoric acid during the course of degumming process. A significant reduction in the oil quality parameters such as P.V., pA.V., and TBARS values were perceived after the degumming process by using 1% phosphoric acid. The most effective adsorbent combination to inflate the PUFA content and diminish the disagreeable color impurities was selected from the various combinations of activated charcoal: Fuller's Earth, and an optimum combination (1.25:3.75, 3%) of these adsorbents was demonstrated. A significant reduction in the content of unsaturation (as in δ 4-5 ppm of ^{1}H-NMR) was obtained by using activated charcoal: Fuller's Earth at the ratio 1.25:3.75 (3%) during the bleaching process. This study further demonstrated that unfavorable fishy odors can successfully be removed by distillation with aqueous acid solution mixture (0.25N acetic acid) (100 ^{o}C) under vacuum. The odor-contributing volatile components such as dodecane, 1-tetradecene, (E,Z)-2,6-nonadienal, (E,E)-2,4-nonadieal, (E,E)-2,4-decadienal, (E)-2-octadecadecen-1-ol, (E,E)-2,4-heptadienal, and (E,E)- 2,4-decadienal in the sardine oil was established as effective flavor quality markers during the process of deodorization leading to the food grade oil. Overall, the extraction method using cooking and pressing followed by degumming process using phosphoric acid (1%) was used in the study. This was followed by bleaching by using the adsorbent combination of activated charcoal-Fuller's Earth (1.25:3.75,

w/w, 3%) and deodorization using distillation process for one hour yielded the refined oil with 40.2, 15, 5.3 and 25.5 % of total SFA, EPA, DHA and total PUFA, respectively. The refining processes considerably reduced the lipid oxidation parameters of the sardine oil as evident from the peroxide, para-anisidine, TOTOX and TBARS values.

Due to the tightly packed structure of saturated fatty acids, negative consequence can occur due to their increased consumption by an increase in LDL cholesterol level and clogging of the arteries. Hence, further purification of the refined oil was performed by an array of purification techniques, which include winterization, urea-complexation and argentated column chromatography. Initially, chemical hydrolysis of refined fish oil was carried out to prepare the sardine oil in the free fatty acid form for subsequent purification steps. The winterization at -20 and 4 ^{o}C of PUFAs by using the organic solvents, such as ethyl acetate and acetone, and their various combinations were performed. An optimum condition of winterization was found out to be in the solvent combination ethyl acetate: acetone (7:3, v/v) at 4 ^{o}C, which yielded the winterized fatty acids with 20.3, 8.91 & 36.1 % of EPA, DHA and total PUFA, respectively. The total SFA content in the winterized oil was reduced to 28.5% during this process. The urea complexation method was performed at three different temperatures (2, 4, and 6 °C) and urea: fatty acid ratios (2:1, 3:1, and 4:1 w/w). The urea complexation at 4 °C using urea: fatty acid ratio 4:1 for 12 h crystallization time has been selected as the optimum process to obtain 33.4, 27.8 and 73.9 % EPA, DHA and total PUFA, respectively in the concentrated fatty acids. The total saturated fatty acids were found to be reduced in the urea complexation process to a minimum of 4.5 %. The urea complexation process was followed by silver-ion liquid column chromatography to completely eliminate the saturated fatty acids. The silver column chromatography with alumina and silica were performed for a comparative study of these adsorbents. The Ag-silica column chromatography using *n*-hexane: acetone was found to be the optimum purification method, and this process showed the maximum recovery of PUFA methyl esters with *n*-hexane: acetone (90:10) fraction (31.6 %). The saturated fatty acid ester was kept

at the level of 0.3 %, with the significantly higher EPA, DHA and total PUFA contents (47.6, 38.2 & 96.7 %, respectively in that order).

The present study also showed the changes in the different classes of fatty acid and composition as a function of time-course (1–6 h) of hydrolysis of triglycerides from sardine oil by a lipase purified from *Bacillus circulans* that may be a potential enzyme source for concentration of *n*-3 PUFAs of acyl chain length shorter than C_{20}. The *n*-3 PUFA (20:5*n*-3) in the triglyceride mixture were found to increase proportionally with the progress of hydrolysis up to 3 h; after that their concentration plateaued as reaction time increased beyond 3 h, due to reduced selectivity of the lipase. It was found that ester bonds of *n*-3 fatty acid homologues were highly resistant to hydrolysis by lipase from *B. circulans*, resulting in their enrichment in fatty acid triglycerides. The urea complexation reaction raised 20:5*n*-3 content by 1.4-fold, thus allowing further purification by argentated chromatography. The fractions eluted with *n*-hexane/acetone (95:5, v/v) and *n*-hexane/ethyl acetate (9:4, v/v), by argentated chromatography furnished 20:5*n*-3 methyl ester in final purities of 60 % of total fatty acids. The lipase-catalyzed reaction offers several benefits, such as greater selectivity and milder reaction conditions. Modification of fats and oils containing PUFAs by lipase may be a process with prospects for enzyme application on a commercial scale.

Fish oil with numerous health benefits is highly vulnerable to the oxidative degradation during and after processing due to the presence of their multiple methylene interrupted *bis*-allylic double bonds in the long chain polyunsaturated fatty acids. The synthetic antioxidants, such as BHT, BHA, propyl gallate etc are conventionally utilized to prevent the oxidation of fish oil and fish oil products. Due to the growing demand for naturally derived antioxidants, the present study focused on the marine sources such as salt marsh halophytes and seaweeds. The significant positive correlation of total phenolic content with antioxidant activities indicated that the phenolic active principles present in these marine additives are endowed with potential antioxidant properties. The ethyl acetate extracts of the halophytes, *Salicornia brachiata* and *Suaeda maritima* were found to possess higher antioxidant

potential as compared to other halophytes when the *in vitro* antioxidant properties are considered. Similarly, the ethyl acetate fractions derived from the crude methanolic extracts of the red seaweeds, *Kappaphycus alvarezii*, *Hypnea musciformis* and *Jania rubens* revealed higher overall antioxidant potential as compared to those of *Gracilaria corticata* and *Hypnea valentiae*. However, *H. valentiae* showed higher content of total phenolics as compared to those in *J. rubens* and *K. alvarezii*, and it also showed better ABTS radical scavenging activity than *J. rubens*. Furthermore, *H. valentiae* exhibited higher hydroxyl radical scavenging activity than *H. musciformis*. Similarly, *G. corticata* showed better ABTS radical scavenging activity as compared to that in *H. musciformis*. It is therefore, that these five candidate seaweeds along with the two halophytic plant extracts, *S. brachiata* and *S. maritima*, were selected to assess the effect of these marine natural additives on lipid oxidation in the refined oil and concentrated sardine methyl esters.

The efficacy of the ethyl acetate fraction of the methanolic extracts of seaweeds, mainly, *K. alvarezii*, *H. musciformis* and *J. rubens* for use as antioxidative supplements to the refined (EPA 14.7 %; DHA 5.7 %; total PUFA 25.8 %), concentrated (EPA 33.4 %; DHA 27.8 %; total PUFA 73.9 %) and purified oil (EPA 47.6 %; DHA 38.2 %; total PUFA 97.3 %) in a time dependent accelerated shelf life study (for 12 days) with respect to the peroxide, para-anisidine, TOTOX, and TBARS values, along with the free radical scavenging activity and fatty acid composition were evaluated. Both refined and concentrated sardine oil were shown to be significantly protected by the extracts obtained from *K. alvarezii*, *H. musciformis* and *J. rubens*. The effect of the seaweed extract blend (especially, COT_3 prepared by mixing 0.1:0.2:0.2 % w/w of *K. alvarezii*, *H. musciformis* and *J. rubens* extracts, respectively) at 0.5 % level to the concentrated oil, showed a positive synergistic effect as calculated from its degree of synergism (% SYN 221.8 %). Furthermore, a threshold level of this blend COT_3 (1 % to purified oil) was demonstrated to be sufficient for optimum antioxidant activity, and further addition of this antioxidant blend (2 % to purified oil) did have no additional improvement on the stability of the essential long chain fatty acids. A significant reduction in the contents of the *n*-3 PUFAs, and deficiency to arrest the isomeric conversion of *cis*

PUFAs to their *trans* isomers of the purified oil were realized, and this was achieved by the addition of the marine antioxidant combination. This combination further realized a comparable antioxidative effect as that of the herbal blend prepared from the rosemary and green tea extracts. The ^{1}H-NMR study further validated that the lipid deterioration of the sardine oil was kept at the minimum and below the threshold level under shelf without additives. The prominent role of the active principles of the marine additives demonstrated their beneficial effect as compared with the synthetic antioxidants, α-tocopherol and BHT. It is therefore, that the sardine oil stabilized with marine additives ensured its nutritional quality by retaining their functional properties for a longer period on shelf.

The natural marine additives were demonstrated to possess potential antioxidant activities, and the solvent extracts derived from the red seaweeds such as *Kappaphycus alvarezii*, *Hypnea musciformis* and *Jania rubens*, were purified by sequential chromatographic process yielding an array of pure compounds, which have been further assayed for their antioxidant activities. The chemical structures of the pure compounds, as well as their relative stereochemistry, were established by means of detailed spectroscopic experiments. The ethyl acetate fractions from the MeOH extract of *Kappaphycus alvarezii*, *Hypnea muscifomis* and *Jania rubens* was found to be the most effective free radical scavenger, which was fractionated chromatographically to yield different compounds with antioxidative activities. The compounds with potential antioxidative activity isolated from *Kappaphycus alvarezii* were found to be (8Z)-3-ethyl-3,4,5,6,6a,7-hexahydro-6-vinyl-10aH-heptaleno[1,10-bc]furan-2,10-dione, which possessed 81.1 % DPPH radical scavenging, 0.99 MDAEQ/kg TBARS formation inhibitory and 3.11 % Fe^{2+} ion chelating activities. The compound, (2Z, 7Z) - methyl 2-ethyl-9-oxo-5-vinyl-1, 4, 5, 5a, 6, 9, 10, 10a-octahydro-1- heptalenecarboxylate., isolated from *K. alvarezii* showed 69.1 % DPPH radical scavenging, 0.11 MDAEQ/kg TBARS formation inhibitory and 41.0 % Fe^{2+} ion chelating activities. 2-(Tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl)ethyl 4-hydroxybenzoate, 2-2-[(4-hydroxybenzoyl)oxy]ethyl-4-methoxy-4-2-[(4-methylpentyl)oxy]-3,4-dihydro-2H-6-pyranylbutanoic acid, and 3-((5-butyl-3-methyl-5, 6-dihydro-2H-pyran-2-yl) methyl)-4-methoxy-4-oxobutyl benzoate were

isolated and characterized from *Hypnea muscifomis*, and were demonstrated to elicit about 50% radical scavenging activity as substantiated by the DPPH radical model. Among these compounds, 2-(tetrahydro-5-(4-hydroxyphenyl)-4-pentylfuran-3-yl) ethyl 4-hydroxybenzoate showed higher Fe^{2+} ion chelating activity (40.2 %). The compound 6-methoxy-4b-methyl-7-vinyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-2-phenanthrenol isolated from *Jania rubens* was found to possess prospective antioxidative properties with 91.4 % DPPH radical scavenging, 0.88 MDAEQ/kg TBARS formation inhibitory and 40.4 % Fe^{2+} ion chelating activities. These antioxidative compounds derived from the candidate seaweed species may potentially contribute towards the scavenging of the free radicals resulting in higher shelf-life of the marine oil with multiple olefinic double bond, which are susceptible towards free radical induced oxidation reactions.

The *n*-3 PUFAs affect diverse physiological processes including cognitive functions and visual acuity, immunosuppressive and anti-inflammatory actions, anti-thrombotic and anti-arrhythmia activities along with their valuable nutritional properties. However, the various methods for the purification of these *n*-3 fatty acids may reduce their bioactive properties due to various factors associated with the sequence of purification process. It is therefore, that the bioactive compounds from marine origin were selected from the class of cephalopods, which have structurally diverse anti-stress metabolites with respect to bioactive properties. The cephalopod species belonging to *Loligo duvauceli* (d'Orbigny, 1835), *Cistopus indicus* (Rapp 1835), *Sepia officinalis* (Linnaeus, 1758), and *Octopus membranaceus* (Quoy & Gaimard 1832), which are abundantly available throughout the coastline of South India were shortlisted to isolate and characterize the bioactive lead molecules. The compounds belonging to pyran derivatives such as 2-(but-3-enyl)-tetrahydro-5-nonyl-2H-pyran and 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3,6-dihydro-2H-pyran were isolated from the ethyl acetate-metahnol (1:1) extract of *Loligo duvacelli*, and were demonstrated to possess potential anti-inflammatory and antioxidative properties. The compound 2-(but-3-enyl)-tetrahydro-5-nonyl-2H-pyran showed potential anti-inflammatory activity as determined by 37 % cycloxygenase-2 (COX-2) and 33 % lipoxidase-5 (5-LOX) inhibition activities (both at 0.5mg/ml), along with more than

65% free radical scavenging activity. The compound 5-(7-butoxydodecyl)-2-(2-ethylbut-3-enyl)-3,6-dihydro-2H-pyran was demonstrated to exhibit more than 30% inhibition in the activities of inflammatory biocatalysts COX-2 and 5-LOX (both at 0.5mg/ml) with 71.7 % free radical scavenging activity. The anti-inflammatory potential of these compounds derived from *Loligo duvacelli* were proved to be comparable to the synthetic anti-inflammatory agents, aspirin and indomethacin.

The evolution of new fish oil products based on refined oil or its concentrated derivatives of specific essential long chain fatty acids are constantly being introduced into the market. A product with enriched anti-inflammatory components was prepared by mixing of purified sardine fatty acid ester with *Loligo duvaucelli* extract. The addition of *Loligo duvaucelli* significantly enhanced the anti-inflammatory property of the purified fatty acid methyl ester, which is evident from the carrageenan-induced paw edema model study in mammalian subjects. Furthermore, the anti-inflammatory activity was found to be significantly higher after 4 h of carrageenan injection. Thus the purified sardine oil with concentrated *n*-3 polyunsaturated fatty acids supplemented with *Loligo duvaucelli* extract has been demonstrated to be the effective alternative to the synthetic non steroidal anti-inflammatory drugs for use against a cascade of mammalian inflammatory diseases.

The *n*-3 polyunsaturated fatty acids in adequate amount are essential in the diet of marine finfish for larval and broodstock nutrition, and as nutraceutical supplements for human health. These essential fatty acids useful for larval and broodstock nutrition in mariculture and human health are widely available in a large variety of marine fish and fishery by products, and therefore, they are preferentially used as raw material to prepare polyunsaturated fatty acid concentrates. Presently the polyunsaturated fatty acid concentrates available in the market are mostly imported and highly priced. Therefore it is not cost-effective to use them as feed or health food additives. The *n*-3 polyunsaturated fatty acid enriched product developed in the present study may be a cheaper alternative to the imported supplements, and will be useful as nutraceuticals, including dietary supplements, and aquafeed products. The *n*-3 polyunsaturated fatty acid enriched formulation from low value "fish body oils"

will also answer the risks associated with hypervitaminosis (A, D) and exposure to environmental toxins (mercury, PCBs, dioxins etc.) associated with the commercially available liver oils. The optimized procedure to prepare the stabilized *n*-3 polyunsaturated fatty acid concentrates developed in the present study would contribute to the commercial application to produce the stabilized formulation of the purified *n*-3 fatty acids from sardine oil, and will be useful as nutraceuticals, including dietary supplements, and aquafeed products.

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LIST OF PUBLICATIONS RELATED TO THESIS

No.	Title of paper	Authors list (As mentioned in the journal)	Volume, P/g no, year	Journal name	National / International (with ISSN /ISBN No.)	Impact factor (Thomson Reuters)	Review or full length article
1.	Concentration and Stabilization of $C_{20–22}$ N-3 Polyunsaturated Fatty Acid Esters from the Oil of *Sardinella Longiceps*.	Kajal Chakraborty, Deepu Joseph, Dexy Joseph	199: 828–837 (**2016**)	Food Chemistry	International **ISSN**: 0308-8146	**3.391**	Full length article
2.	Characterization of Substituted Aryl Meroterpenoids as Potential Antioxidants to Polyunsaturated Fatty Acids	Kajal Chakraborty*, Deepu Joseph, Vamshi Krishna Raola, Minju Joy	Accepted manuscript (**2016**, in press)	Food Chemistry	International **ISSN**: 0308-8146	**3.391**	Full length article
3.	Production and Characterization of Refined Oils Obtained from Indian Oil Sardine (*Sardinella longiceps*)	Kajal Chakraborty, Deepu Joseph	63(3):998-1009 (**2015**)	Journal of Agricultural and Food Chemistry	International **ISSN:** 0021-8561	**2.857**	Full length article

4.	Antioxidant Activities And Phenolic Contents Of Three Red Seaweeds (Division: Rhodophyta) Harvested from the Gulf of Mannar of Peninsular India	Kajal Chakraborty, Deepu Joseph, & N. K. Praveen	52(4): 1924-1935 (**2015**)	Journal of Food Science and Technology	International ISSN: 0022-1155	**2.203**	Full length article
5.	Concentration of C_{20-22} n-3 polyunsaturated fatty acids from *Sardinella longiceps* and their stabilization	Kajal Chakraborty, Deepu Joseph, Dexy Joseph	118, 208–223 (**2016**)	European Journal of Lipid Science and Technology	International **ISSN**: 1438-7697	**1.953**	Full length article
6.	Cooking and pressing is an effective and ecofriendly technique for obtaining high quality oil from *Sardinella longiceps*	Kajal Chakraborty, Deepu Joseph	117: 837–850 (**2015**)	European Journal of Lipid Science and Technology	International **ISSN**: 1438-7697	**1.953**	Full length article

7.	Seasonal and inter-annual lipid dynamics of spiny cheek grouper *Epinephelus diacanthus* in the southern coast of India.	Kajal Chakraborty, Deepu Joseph, Selsa J. Chakkalakal	94(8), 1677–168 (**2014**)	Journal of Marine Biological Association of the United Kingdom	International ISSN: 0025-3154	**1.064**	Full length article
8.	Changes in the quality of refined fish oil in an accelerated storage study	Kajal Chakraborty, Deepu Joseph, Dexy Joseph	Accepted manuscript (in press) **,** Published online: 26 April, **2016** DOI:10.1080/10498850.2015.1036482	Journal of Aquatic Food Product Technology	International ISSN: 1049-8850	**0.688**	Full length article
9.	Inter annual and seasonal dynamics in lipidic signatures of *Trichiurus lepturus.*	Kajal Chakraborty, Deepu Joseph, Selsa. J. Chakkalakal, P.S. Stephy	Accepted manuscript (in press)**,** Published online: (29 Dec **2015)** DOI:10.1080/10498850.2014.935541	Journal of Aquatic Food Product Technology	International ISSN: 1049-8850	**0.688**	Full length article
10.	Inter annual and seasonal dynamics in lipidic signatures of *Sardinella longiceps.*	Kajal Chakraborty, Deepu Joseph, Selsa. J. Chakkalakal	Accepted manuscript (in press) **,** Published online: (06 March **2015)** (DOI:10.1080/10498850.2014.895918)	Journal of Aquatic Food Product Technology	International ISSN: 1049-8850	**0.688**	Full length article

11.	Inter-annual variability and seasonal dynamics in lipid signatures of *Leiognathus splendens* (Cuvier, 1829)	Kajal Chakraborty, Deepu Joseph, S. J. Chakkalakal	21(4): 1699-1706 (**2014**)	International Food Research Journal	International ISSN 19854668	**0.200**	Full length article
12.	Halophytes of Chenopodiaceae and Aizoaceae from South-East coast of India as potential sources of essential nutrients and antioxidants	Deepu Joseph, Kajal Chakraborty, CS Subin, KK Vijayan	1 (5): 97-107 (**2013**)	Journal of Food and Nutrition Research	International ISSN 2333-1119	-	Full length article
13.	Interannual and Seasonal Dynamics in Amino Acid, Vitamin and Mineral Composition of *Sardinella longiceps*.	Kajal Chakraborty, Deepu Joseph, Selsa J Chakkalakal, & Koyadan Kizhakedath Vijayan. (2013).	1(6), 145-155 (**2013**)	Journal of Food and Nutrition Research	International ISSN 2333-1119	-	Full length article